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MULTILOCUS VNTR ANALYSIS DISCLOSES AN UNEXPECTED INTRAPATHOVAR VARIABILITY OF *PSEUDOMONAS SYRINGAE* PV. *ACTINIDIAE* WORLDWIDE

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SUMMARY

1. ABSTRACT

Nowadays, kiwifruit bacterial canker is a worldwide spread disease affecting all the major producer countries; *Pseudomonas syringae* pv. *actinidiae* (Psa) is well-known to be its etiological agent: nonetheless so far a definitive cure doesn't exist.

Traditionally Psa has been thought to be made up of 4 populations, according to macroareas of isolation and virulence degrees. Scientific community has come to this classification using several, often laborious and expensive, methodologies such as genome-wide SNPs analysis, whole genome sequencing and MultiLocus Sequence Analysis (MLSA). However the source of the pandemy begun in 2008 has not been yet definitely determined.

To understand more in depth population structure, epidemiology and mode of transmission of Psa, a MultiLocus Variable Number Tandem Repeats (VNTR) Analysis (MLVA) was set up and validated on a large collection of Psa strains, as much as possible representative of worldwide population. MLVA scheme has been made up of two different panels, comprising a total of 13 VNTR loci: the "diversity panel", including 6 loci, has been used to perform the preliminary analysis providing results consistent with previous 4 populations-model; the whole 13 loci panel showed to be very useful to deeply clarify Psa population structure at high resolution level.

Two several types of elaboration, MST and UPGMA, were carried out to check the reliability of the established MLVA system, both showing similar and consistent results.

The analyzed collection was found to correspond to 11 clonal complexes plus 5 singletons, revealing a genetic variability never imagined before. The most diversified group resulted to be the Chinese one encompassing 6 different clonal complexes, one of these shared with all the strains related to 2008 outbreak, constituting a very homogeneous group: these data strongly suggest that the recent pandemy has been originated from this area. South Korea and Japan populations showed to be a very variegated cluster with at least two different and chronologically separated populations and surprisingly the only Turkish strain included in the study belonged to one of the Korean clonal complexes. Moreover, as proposed by some scientists and as deducted by MLVA, Psa-LV should be reassign to a new pathovar, since it shares with all the other Psa-V a common profile for only 2 on 13 loci.

Key words: *Pseudomonas syringae* pv. *actinidiae*, Variable Number Tandem Repeats, MultiLocus VNTR Analysis, Epidemiology, Clonal Complex.

2. INTRODUCTION

2.1 Bacterial canker of Kiwifruit: history, symptoms and defense strategies

Kiwifruit (*Actinidia* spp.) is native to Southern China where it has been cultivated since at least 700 years for its fruits and for ornamental aim too. At the beginning of the Nineteenth century it came to England, and in the Twentieth century it spread through intensive farming in New Zealand, where it found a quite favorable environment. At the end of the Twentieth century it spread in Europe and especially in Italy, where Bollettini from San Benedetto del Tronto exported the fruit first (http://www.zipmec.com/kiwi-storia-produzione-commercio-guide-frutta.html).

According to the data of FAOSTAT, except for China for which no official data are available about the real volumes of the installations and productions, nowadays in the world the surface dedicated to the culture of kiwi in 2012 was close to 99.000 hectares, whereas it was of 54.000 at the end of the Nineties. Chinese production turns around 450.000 tons.

Regarding to production surface, among the Northern hemisphere countries, Italy stands out (24.800 ha in 2014), followed by Greece (7.300 ha in 2012), and France (3.952 ha). In the Southern countries the culture is concentrated in New Zealand (12.757 ha) and in Chile (11.000 ha). Italy, with 408.800 tons for 2013/2014 and 419.000 tons estimated for 2014/2015 years, produces 65% of kiwi in the Northern hemisphere, affirming its world leader position. According to the forecasts of the CSO and IKO the 2014/2015 production should increase by 3% compared to the former season, but on the other hand drop on the average of the 2009/2012. An increase in the production is also planned for Greece which is the second producer of Europe exporting 80% of its supply because of the reduced internal market demand. Greece will increase of +32% (125.000 tons), France +7% (60.000 tons), and Spain +12% (12.500 tons). A fall of production is planned for Portugal (-25%, 16 tons), California (-10%, 21.400 tons) and South Korea (-15%, 11.500 tons). In general, the estimated production is in the average of the last 5 years, registering an increase of 6% compared to the previous year. According to FAOSTAT, in the Northern hemisphere there is a strong increase in the Turkish production which passed from 26.554 tons in 2010 to 36.781 in 2012. Surprisingly, Iran increased its production with an average of 32.000 tons in 2012, compared to the 20.000 tons in the

first years of 2000. The production of Iran is concentrated in the North of the country, with a total production of 99% in the Hayward zone. The production increased especially in 2013 when the government removed export restrictions enabling a competitive market with lower economic kiwi prices compared to New Zealand and Chile.

On a worldwide level, New Zealand is the country which exports the most of its kiwifruit production, with 90% followed by Europe with 80% of exports. Italy exports 70% (+1%) compared to 2013, and France its 33%. The Far East exports 50%, while Chile underwent a fall of 49% compared to the previous years (source: www.areflh.org).

Probably the most harmful and lethal pathology of kiwifruit is the bacterial canker: it is a severe disease leading as final result to plant death by wilting and characterized by symptoms becoming firstly visible in spring/early summer, as foliar brown necrosis surrounded by yellow chlorotic haloes, collapsed fruits and flowers, brown coloration of buds (Fig. 1); during the summer the disease seems to slow down. The most typical and characteristic sign appears in the final stage of the disease in late winter and it is represented by whitish to reddish exudates which ooze from branches and trunks: now it is well-known that the exudates are one of the strategies that Psa exploits to rapidly spread between plants and orchards (Koh, 2010; Scortichini, 2012). Both most commercially relevant species of kiwifruit, *Actinidia deliciosa* and *A. chinensis*, are targeted by this pathology, together with less known species and variety, particularly present in China of which kiwifruit is native of (Vanneste, 2014).

Etiological agent of bacterial canker of kiwifruit was firstly isolated in Japan and named *Pseudomonas syringae* pv. *actinidiae* in 1989 (Serizawa, 1989; Takikawa, 1989), even if one case was previously reported in the Chinese province of Hunan in 1984 (Fang, 1990); subsequently, the same disease was observed also in South Korea (Koh, 1994). A few years later, in 1992 the same symptoms on kiwifruit appeared in Italy (Scortichini, 1994), where a new and stronger outbreak of bacterial canker occurred on *Actinidia chinensis* (Balestra, 2008): hence, the disease spread very rapidly in Turkey (Bastas, 2012), France (Vanneste, 2011a), Portugal (Balestra, 2010) and New Zealand (Everett, 2010) in 2010; then in 2011, kiwifruit orchards in Switzerland (Service ER, 2011), Spain (Balestra, 2011), Australia (Australia B., 2011) and Chile (Anonymous, 2011) were affected by bacterial canker. The latest countries to be hit were Slovenia (EPPO Reporting Service, 2014) and Germany in 2013, even if here the contagion was delimited just within two areas (EPPO Reporting Service, 2013), and Greece in 2014 (Holeva, 2015) (Fig. 2).

So fast and wide diffusion of Psa reaching pandemy level in a few years, leaves to infer how little was initially known about vectors, modes and routes of transmission; in the last decade, many studies have focused on this side aiming to successfully prevent the pathogen overflowing. Besides asymptomatic vegetative material (Deflorian, 2009), also pollen was found to be a possible carrier for Psa as it can survive on it for a quite long time (Vanneste, 2011b; Tontou, 2014); fruits too were thought to be potential responsible for transmission, but deep investigations on epyphitic pathogen survival on them made refuse the hypothesis (Balestra, 2014a).

Nowadays, there is not yet a definitive and infallible cure to kiwifruit bacterial canker and the awareness that the only way to face the problem is the coexistence with Psa is becoming stronger and stronger. However, besides correct agronomic and culture practices, such as disinfection of pruning tools, closing of natural or artificial wounds by mastic or natural waxes, burning of pruning remains and infected plant portions, which help to contain infected zones, years-lasting researches have shaded light on several and important aspects of pathogen and so on the possible strategies to manage it.

Antibiotics use is forbidden in agriculture within European Community, whilst in other countries they are regularly distributed on orchards. New Zealand has been tried to face Psa by using essentially kasugamycin- and streptomycin-based pesticides (respectively Kasumin® and KeyStrepto™) but with no satisfactory results, since on April 30th 2015 87% of New Zealander kiwifruit cultivations, corresponding to 2.724 orchards and 12.009 hectares, were recorded to be infected by Psa (source: www.kvh.org.nz). Similar critical situations could be observed in Japan, Korea and Chile. In addition to the lack of clear advantages in field conditions, it has already been ascertained that Psa is able to acquired antibiotics resistance (Han, 2003; 2004).

Copper and copper-based compounds have traditionally been used against bacterial crop pathogens since their broad-spectrum antimicrobial properties (Balestra, 2006). Nevertheless, European Community is trying to reduce the use of chemicals, including copper, in agriculture management, searching for more human health- and eco-friendly defense strategies. In the case of Psa, copper is recommended in many kiwifruit protection plans, but, as for streptomycin, there are clear evidences of potential resistance of Psa to it, already expressed in some Japanese strains isolated in the late Eighties (Nakajima, 2002). By virtue of these aspects, many efforts have been addressed to the research of biocontrol agents (BCAs) useful to contain and to manage kiwifruit bacterial canker pathogen (Mathre, 1999). Interesting results were pointed out by studies involving natural extracts

(Monchiero, 2015), selective bacteriophages (Di Lallo, 2014) and antagonistic microorganisms; among them, *Bacillus amyloliquefaciens* subsp. *plantarum* D747 was proven to be very effective in competing with Psa (Biondi, 2012); in the field it showed a good preventive activity. At the moment it is registered specifically for Psa management under the commercial name of Amylo-X® (Balestra, 2014b).

Fig. 1 Symptoms of bacterial canker on kiwifruit. A) collapsed fruits; B) brown leaf spots with chlorotic halo; C) reddish exudate from branch; D) necrotic buds; E) kiwifruit tree wilting.

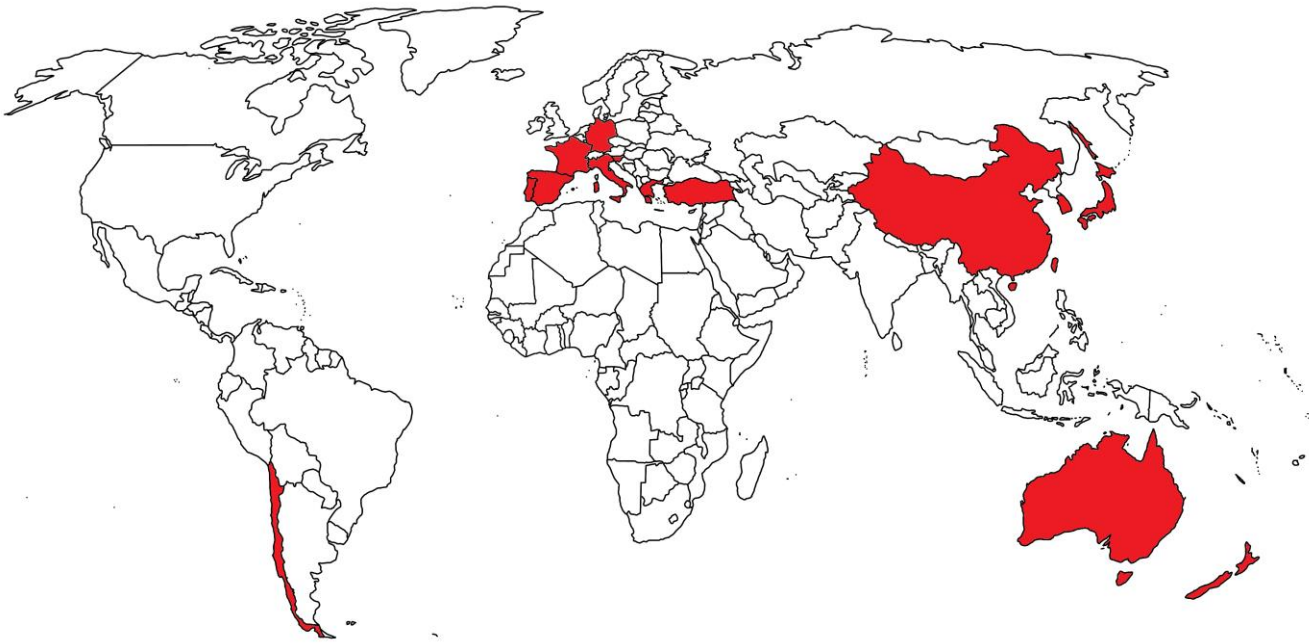


Fig. 2 Current distribution of bacterial canker of kiwifruit worldwide.

2.2 *Pseudomonas syringae* pv. *actinidiae*

Pseudomonas syringae "sensu latu" represents one of the most articulated bacterial species complex that includes 9 recognized plant pathogenic species, which are *P. cannabina*, *P. avellanae*, *P. amygdali*, *P. ficuserectae*, *P. savastanoi*, *P. tremae*, *P. meliae*, *P. caricapapayae* and *P. syringae* (ISPP Taxonomy of Plant Pathogenic Bacteria Committee, http://www.isppweb.org/names; Young, 1978).

Concerning last species *P. syringae*, it represents in turns another wide and heterogeneous complex of different taxa, among which several are dangerous pathogens, named pathovars, in relation to their preferences in attacking specific hosts. More than 60 are actually recognized (Young, 2010), whilst many other strains genetically referable to *P. syringae* are merely environmental bacteria.

P. syringae is responsible for many diseases on crop, forest and ornamental plants, whose typical common symptoms are leaf spot, branches wilting, exudates-producing canker (Hirano, 1990).

Due to its extent, *P. syringae* is characterized by great heterogeneity for as regards molecular aspects (Gardan, 1992; Sawada, 2002). The most recent and commonly recognized intraspecies classification is mainly based on sequences of housekeeping genes, components of "core genome" and not so inclined to horizontal transfer: they reveal that *P. syringae*, despite "flexible genome" complexity, shows to be highly clonal referring to genes essential for bacterial survival, which result poorly related to host plant, that is to pathovar (Fig. 3) (Sarkar, 2004).

Psa is one of the pathogenic group belonging to *P. syringae* complex inside genomospecies 8, responsible for bacterial canker of kiwifruit; it is a Gram negative, aerobic, non-sporing, rod-shaped and motile with one to three polar flagella bacterium (Takikawa, 1989). It can enter its host through natural openings, such as stomata and lenticels, and through wounds caused by atmospheric agents (Ferrante, 2014) or anthropic interventions: once inside the plant, Psa tends to migrate towards crown to cause the damages above described; it has been also demonstrated that Psa can invade and destroy both phloem and xylem, where it induces tyloses formation and that it can overwinter in root apparatus, making useless tree topping practice (Renzi, 2012).

Until recently, according to virulence degree on host and pathogenicity tests, recognition of two different populations of Psa has been broadly accepted: Psa-V (Virulent) has been considered the true etiological agent of kiwifruit canker causing all of the symptoms described in the previous paragraph, whilst Psa-LV (Low Virulent) induces the appearance of just foliar spots with chlorotic haloes but not the other signs, so it doesn't lead plants to death. Right now, the LV population is present in Australia, France and New Zealand, where it coexist together with the V population (Vanneste, 2013). Founding on biochemical, pathogenicity and genetic characteristics, it has been recently proposed to assign LV population to a different new pathovar (Butler, 2013; Cunty, 2014; Ferrante, 2015).

At this moment, many DNA-based protocols for Psa detection even on asymptomatic samples are available. The first attempt to get a reliable detection method was set up by Koh and Nou and was founded on the amplification of a pathovar-specific genomic DNA sequence giving a 492 bp single amplicon (Koh, 2001), although it revealed some specificity problems (Scortichini, 2002). 16S-23S rDNA intertranscribed spacer region was targeted too for early and accurate diagnosis of Psa, but the greatest limit was maybe represented by low sensitivity, which drew attention to the need of a preliminary process such as an enrichment or isolation step (Rees-George, 2010). Low specificity and sensitivity difficulties were ridden over by studying and validating a duplex-PCR test based on

Koh-Nou marker and an amplicon located on *avrD1* gene: the protocol not only showed to differentiate between Psa and the most similar groups *P. syringae* pv. *theae*, *P. avellanae* and *P. s.* pv. *tomato*, but also to be efficient working on spiky matrices, such as plant tissues and pollen (Gallelli, 2011). A further improvement was reached in 2012 when a multiplex PCR assay was established: four primer pairs, named Psa, Europe, China and Japan/Korea, could be simultaneously used in one reaction of amplification, allowing to distinguish Psa from closest related Pseudomonads and at the same time correctly assign recognized Psa to haplotype, and so to its geographic origin (Balestra, 2012). Recently, also Real Time PCR protocol based on *hrpW* gene was used to detect and to quantify Psa-V presence in whatever matrices (Gallelli, 2014).

Since the beginning of the last massive and harmful epidemic, different types of DNA-based analysis were carried out attempting to characterize genetically the populations of Psa (Lee, 2005). One of the first attempts in such sense was the repetitive PCR (rep-PCR) analysis, based on the use of primers BOX, ERIC and REP, which gives specific banding patterns according to species or even strains, providing information about genomic rearrangement (Louws, 1994; Ferrante, 2010; Mazzaglia, 2011). The first indications obtained in these researches were about differences between the groups of European, Korean and Japanese strains. Then, one of the most popular technique for phylogenetic studies of bacterial population, the MultiLocus Sequence Typing (MLST) was repeatedly applied to study Psa. As example, Chapman and his group amplified, sequenced and analyzed seven housekeeping genes (*acn*, *rpoD*, *gapA*, *cts*, *pgi*, *pfk*, *gyrB*), eleven putative effector genes and phytotoxin genes and resulted able to allocate different Psa strains into 4 MLST groups: Psa-1 for strains from Japan and the Italian strain obtained in 1992 report; Psa-2 for Korean strains; Psa-3 for New Zealander, Chinese, Chilean and Italian (after 2008) strains; Psa-4 for LV population (Fig. 4) (Chapman, 2012). Then, since 2011, some research groups have started approaching genetic analysis of Psa by genome analysis. Three main papers figured out a more detailed picture of the genetic variability present in Psa populations worldwide. In a first paper, Mazzaglia and colleagues sequenced 9 strains of Psa and 1 of the pathovar *theae*, known to be the genetically closest to Psa. Interestingly, an almost complete correspondence was found between the 3 Chinese strains, isolated from Shaanxi province in 2010, and European strains, separated each other by only 6 SNPs, but with some noteworthy differences in a particular genomic island (PPHGI-like). The genomic analysis confirmed that these strains are clearly distinct from Japanese and Korean strains. A derived PCR approach, in the same paper, indicates that also the virulent strains from New

Zealand belong to the Chinese/European group (Mazzaglia, 2012). In 2013, Butler and colleagues investigated the genome of 23 Psa strains and, focusing mainly on the analysis of genomic islands (Integrative Conjugative Elements - ICE), they shed light on a plausible, independent Chinese origin for each European, New Zealander and Chilean outbreaks; this research also suggests that low virulent strains are merely not true Psa (Butler, 2013). The most recent paper on Psa genomes, taking in account 34 Psa draft genomes and 2 complete circular genomes, confirms the existence of 4 different Psa lineages, debates in deep the significance of these differences, again mainly related to pathogenicity islands, and suggests a common, recent ancestor for these lineages (McCann, 2013). The last year, a new MLSA group named biovar 5 was isolated and identified in Japan (Sawada, 2014).

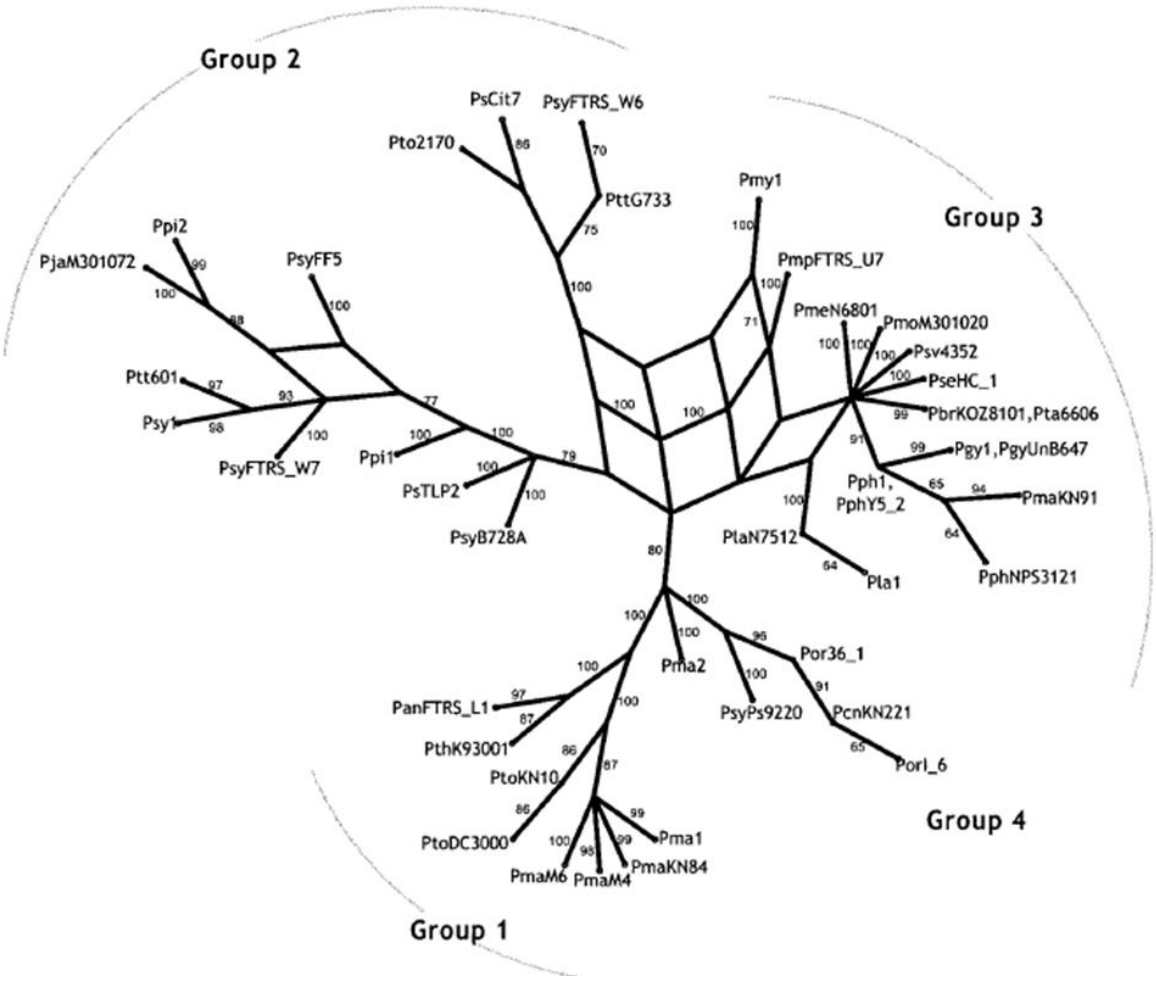


Fig. 3 *P. syringae* complex as obtained by 7-genes MLST; Psa is indicated as PanFTRS_L1 in group 1 (Sarkar, 2004).

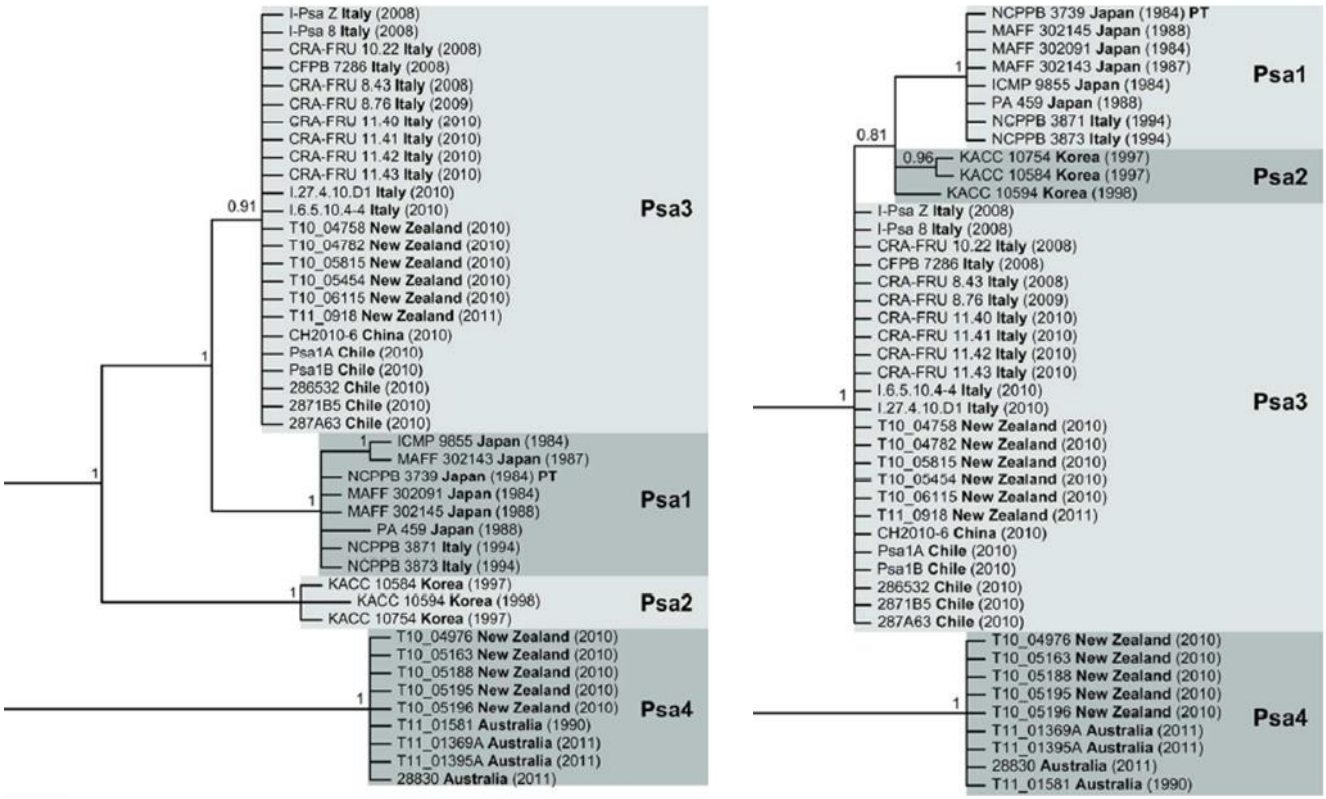


Fig. 4 The 4 haplotypes recognized within Psa population according to MLSA on housekeeping (A) and effector (B) genes (Chapman, 2012).

2.3 MultiLocus Variable Number Tandem Repeat Analysis

In the last years, the value of repeated sequences markers (mini satellites, microsatellites, etc.) in highlighting coherent differences between individuals of a same taxa has been clearly demonstrated. In particular, the chance to identify different clonal populations within strictly delimited taxa of several human bacterial pathogens was attained by means of a molecular technique named MLVA (Multiple Locus VNTR Analysis) (Linstedt, 2005). This technique relies on the detection of length variations of specific DNA sequences; the differences are ascribable to

variations in the number of copies of well-defined repeated units: these sequences are known as Variable Number of Tandem Repeats (VNTR) (Fig. 5a). The amplicons, obtained by standard PCR using primers designed on both the flanking regions of the Tandem Repeat (TR), are analyzed by high resolution electrophoresis techniques (i.e. capillary electrophoresis) or by high resolution melting curve analysis (HRMA) (Ciammaruconi, 2009). The VNTRs originate from DNA polymerase slippage or from recombination events during DNA duplication and are subjected to a different selective pressure depending on whether they lie within coding or non-coding regions and depending on repeat unit size; so, different (for position and size) types of VNTR can provide different variability rates (Pourcel, 2011a). Once a panel of proper VNTRs is defined for a specific organism (Fig. 5b), the MLVA provides many advantages, such as being faster, cheaper and less time- and labor-consuming in comparison to other comparable techniques, like PFGE or AFLP (Sawada, 2002); moreover, combining MLVA with capillary electrophoresis provides very high resolution data.

The first approach to this method was carried out by Andersen and colleagues, that found a sequence of twelve base pairs, differing in number of repeats, able to distinguish *Bacillus anthracis* from the closely related species *B. cereus* and *B. mycoides* (Andersen, 1996); on the same bacterium another VNTR sequence that permitted to differentiate strains into 5 allelic types with strict connection to their geographical origin was later discovered (Jackson, 1997). More recently, optimized HRMA was applied on 6 of 25 loci tested for MLVA feasibility on *Bacillus anthracis*, improving the speed and reproducibility of analysis (Fortini, 2007). Then, VNTR-based approach was applied to other bacteria of clinical interest: serotypes of the food-borne bacterium *Listeria monocytogenes* was subtyped using this new method, as part of a routine surveillance program (Murphy, 2007); in addition to SNPs method, it was used to classify *Yersinia pestis* in Madagascar, identifying patterns relative to geographically separated subpopulations and helping to assess long distance transfer of the pathogen using humans as vehicle (Vogler, 2011). VNTR-based method was then applied successfully on many other noteworthy human bacterial pathogen such as *Brucella spp.* (De Santis, 2009; Garofolo, 2013), *Pseudomonas aeruginosa* (Naze, 2010; Sobral, 2012) and *Mycobacterium tubercolosis* (Frothingham, 1998; Supply, 2006; De Beer, 2012).

So far, just few cases of MLVA application on phytopathological bacteria are known.

Della Coletta-Filho and his group studied VNTRs on *Xylella fastidiosa* genome (Della Coletta-Filho, 200); on *Xanthomonas citri* pv. *citri*, it was moreover demonstrated that MLVA has an

higher resolution degree than AFLP or IS-LM-PCR (Insertion Sequence-Ligation Mediated-PCR; Bui Thi Ngoc, 2009); also in the case of *Xanthomonas arboricola* pv. *pruni*, MLVA revealed to be much more effective than the other most known typing methods, as AFLP or rep-PCR (Bergsma-Vlami, 2012); Zhao and colleagues have developed a VNTR-based protocol to study the rice pathogen *Xanthomonas oryzae* pv. *oryzicola*, thus managing to discriminate between Asian and African strains (Zhao, 2012); also on *Ralstonia solanacearum*, the causal agent bacterial wilt on many crops, was successfully approached MLVA aiming to distinguish populations related to different hosts (N'Guessan, 2013). *Pseudomonas syringae* pv. *maculicola* was in depth analyzed in relation to *P. s.* pv. *tomato*, using both sequencing of housekeeping genes and eight variable number TRs, with highly consistent results between them (Gironde, 2012). Another example of application of MLVA technique on phytopathogenic bacteria concerns *Clavibacter michiganensis* subsp. *michiganensis*, and its effectiveness was demonstrated in deciphering differences between strains from recent Belgium outbreaks in 2010-2012, and others worldwide collected (Zaluga, 2013): also in this case, the MLVA method showed to be the most efficient in comparison to other techniques.

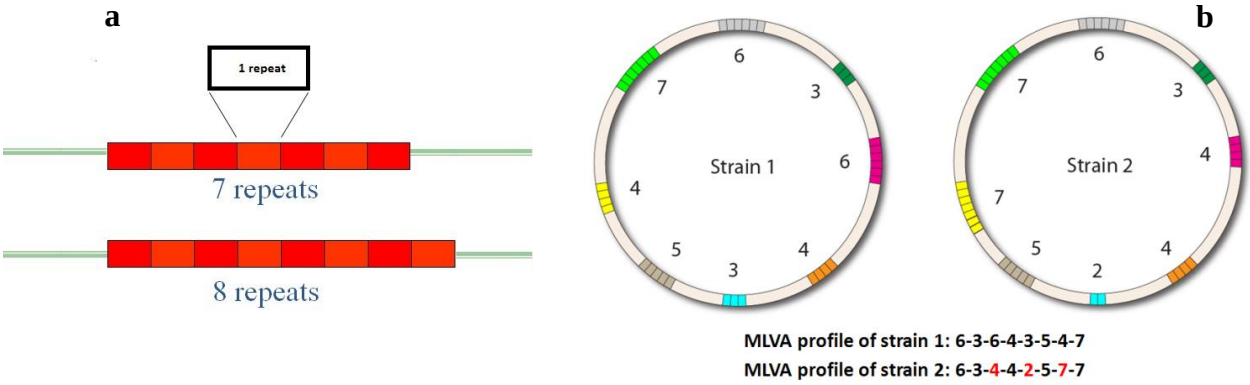


Fig. 5 Single VNTR differing for repetition number (a) and several VNTRs dispersed in the genome, generating a hypothetical MLVA panel (b).

3. AIM OF WORK

The approaches described into introduction have given a lot of new detailed evidences on populations of Psa, but the lack of a congruous number of strains from China, the main area of origin of *Actinidia* species, probably jeopardizes the chance to acquire additional information on where this pernicious pandemic comes from.

So, further researches are needed to unravel what still remain unclear, most of all about the possible diversities among Psa populations in the enormous area of *Actinidia* cultivation in China, where there is also the natural presence of hundreds of wild *Actinidia* species.

Another topic that can deserve further attention is directly related to the WGS analysis; as a matter of fact, for its own methodology that assume the overlapping of small reads ends, the WGS sequences reconstruction inexorably fails to give almost any evidence about a known powerful source of differential information, as the repeated sequences are.

In this study possible differences between strains of different geographical origins, isolation dates, host species and virulence were investigated, in order to individuate clearly and promptly the sources of infection and to break out immediately any further diffusion. In such sense, the successful history of the MLVA technique reported above has incentivized the search for an incisive VNTRs panel for Psa and to test it on a truly exhaustive collection of Psa strains, coming from nearly all the worldwide areas where the pathogen is already established, aiming to assess so far unknown information about Psa intrapathovar relationships, and attempting to trace their origin and routes of transmission.

4. MATERIALS & METHODS

4.1 *In silico* search of candidate VNTRs and primers design

3 WGS genomes of Psa, differing for geographic origin and isolation year, were selected and downloaded as FASTA files from NCBI (https://www.ncbi.nlm.nih.gov). They were converted into concatenated form and submitted to Tandem Repeat Database by Gelfand and colleagues (https://tandem.bu.edu/cgi-bin/trdb/trdb.exe; Gelfand, 2007) to search Tandem Repeats (TRs) applying Tandem Repeat Finder (TRF) algorithm (Benson, 1991); the following parameters were set up:

- Minimal alignment score: 50
- Match, mismatch, indels: 2, 7, 7
- Maximal period dimension: 500

So-found TRs were later compared by means of Position Comparison Tool on the same site, aiming to point out those sequences occupying the same position on two genomes but repeated a different number of times: at this point good VNTR candidates were selected. Right and left flanking regions of 100 bp were individuated and blasted against NCBI databank to check whether they were present in all of the deposited WGS Psa genomes; subsequently they were used to design primers pairs (http://www.bioinformatics.nl/cgi-qbin/primer3plus/primer3plus.cgi) to amplify each candidate VNTR and they were aligned again by Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) on NCBI to ensure a single PCR product.

Once the MLVA panel was composed, each VNTR locus was searched on WGS reference genome of M302091 strain (NCBI WGS Project AEAL01) to locate the position and to define the putative function of associated open reading frames (ORFs).

4.2 Bacterial culture conditions

Selected Psa strains were retrieved from -80°C freezer, streaked on King B plates (peptone 20 g; glycerol 10 ml; K_2HPO_4 1.5 g; $MgSO_4 \cdot 7H_2O$ 1.5 g; technical agar 15 g; deionized water 1 l) and let grow at 28°C for 48 hours; then from each plate genomic DNA was extracted. The whole set of isolates involved in the study is listed in table 2; their identification was preliminarily confirmed by Koh (Koh, 2001) and Rees-George (Rees-George, 2010) specific PCR. The 10 isolates marked with † constituted the screening group of Psa, since they are characterized by different site and date of isolation: PSA_7286, Italy 2008; PSA_830, Spain 2011; PSA_832, Portugal 2011; PSA_2F, France 2010; PSA_K2T, Turkey 2011; PSA_18875, New Zealand 2011; PSA_23664, South Korea 1989; PSA_K2, South Korea 1997; PSA_CH2010-6, China 2010; PSA_JILO4, China 2012.

4.3 Genomic DNA extraction

Once grown, genomic DNA was extracted from every plated Psa isolate by DNeasy Plant Mini kit (QIAGEN, Milan, Italy). DNA samples were quantified using Qubit™ Fluorometer (Invitrogen, Life Technologies Italia, Monza, Italy), then dilution buffer was added to reach for each sample 40 ng/µl concentration. So-prepared samples were kept at -20°C until they were used.

4.4 Amplification by Polymerase Chain Reaction (PCR)

VNTRs candidates selected to be tested on Psa screening isolates (marked by † in table 2) are listed in table 1. Beside sequences found by analysis on TRDB site, 10 VNTRs coming from Gironde & Manceau work (Gironde, 2012) were included in MLVA panel to evaluate their potentiality for Psa intrapathovar analysis: they are named GMxxx in table 1; in this case, primer pairs reported in the paper were used. All the primers couples were ordered and bought from Macrogen (Macrogen Europe, Amsterdam, the Netherlands). Amplification protocols were set up on a C1000 thermal cycler (Biorad Laboratories Inc., CA, USA) with the following steps: initial denaturation at 94°C

for 5 minutes; then 30 cycles made up of denaturation at 94°C for 30 seconds, annealing temperature depending on specific primers pair (Tab. 1), elongation at 72°C for 45 seconds; final elongation at 72°C for 7 minutes. Every reaction mix was prepared as follows: 12.5 µl of GoTaq Colorless Master Mix 2X (Promega Corporation, Madison, WI, USA) containing GoTaq® DNA Polymerase, 400 µM of each dNTP, 3mM $MgCl_2$; 1 µl of genomic DNA sample (40 ng); 1 µl of forward primer and 1 µl of reverse one corresponding to 10 µM concentration each; 9.5 µl of nuclease-free water to reach the final volume of 25 µl. The reactions were prepared on the same way for VNTRs amplification passing the screening phase on the remaining Psa isolates.
All of the experiments were performed twice.

4.5 Amplicons analysis using capillary electrophoresis

PCR products were analyzed by capillary electrophoresis apparatus QIAxcel (QIAGEN, Milan, Italy) to find out their accurate dimensions; default method OM700 of High Resolution Cartridge was chosen and thus the following parameters were applied: injection phase at 5 KV for 10 seconds; separation phase at 3 KV for 700 seconds. Only amplicons coming from Psa-9 amplification, characterized by a greater dimension, were run using OM500 default method, comprising a separation phase of 5 KV for 500 seconds. OM700 parameters allow to discriminate PCR products differing for only 3-5 bp. Results were analyzed by Screengel software (QIAGEN), which gave accurate values for both size and concentration of the amplicons. During the screening phase, primers giving a PCR product of identical length for all the strains were discarded and not involved in the subsequent procedure of the entire Psa collection analysis.
All the process was repeated twice.
The whole set of PCR products belonging to three representative Psa strains (CFBP 7286 Italy, CH2010-6 China, KW11 Japan) were sequenced, together with the ones difficult to assign because of a poorly clear repetition number or discrepancies in WGS alignment.

4.6 Statistical elaboration of data

Once the accurate dimension of amplicons of the 168 Psa strains for every VNTR was found out, associated TR repetitions number was calculated for each of them, applying the following formula (all the variable are expressed in bp):

$$\boldsymbol{n = \frac{x - (a + b + pT)}{T}}$$

Where:

- *n* is the repetition number of TR;
- *x* is the amplicon dimension as obtained from capillary electrophoresis;
- *a* and *b* are the dimensions of respectively right and left flanking regions;
- *pT* is the dimension of the latest repetition (often it is truncated);
- *T* is the repeated unit length.

Repetition number, if the latest repetition is truncated, was conventionally rounded up to the upper integer number, whilst the lack of amplification was codified as -1. The final outcome of the process is constitute by a MLVA profile, that is a numerical string, specific for every considered Psa strain. For the subsequent elaboration, VNTR loci were grouped into two panels characterized by differential variability degrees and consecutively applied for the analysis of the whole Psa set: the first one made up of loci giving low number of haplotypes, named "diversity panel", and the second one composed by the entire set of 13 VNTR loci.

Simpson's (Simpson, 1949) and Hunter-Gaston's (Hunter, 1988) diversity indices were calculated for every single VNTR locus by means of online tool V-DICE (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl).

The VNTR alleles were then analyzed as "character" data using Bionumerics 7.1 (Applied Maths NV. http://www.applied-maths.com.); the data, inserted as "categorical" data matrix and given an equal weight to all the VNTRs, were used to build Minimum Spanning Tree (MST) and Unweighted Pair Group Method with Arithmetic means (UPGMA) graphics.

MST allows to draw a clear scheme of the evolutionary relatedness between haplotypes by comparison of allelic profiles. The criterion of 33% of differences on the total number of VNTR loci (2 mismatches on 6 loci) for analysis with "diversity panel" and of 23% (corresponding to 3

mismatches on 13 loci) for the whole MLVA panel were established to group haplotypes into clonal complexes (CC). Clonal complexes are each other related according to the increasing number of mismatches between haplotypes.
Furthermore, a similarity matrix was elaborated to build UPGMA dendrogram where the percentages reflect the percentage of homology between specific markers, for each macrogroup.

4.7 *In vitro* stability test

PSA_7286 was chosen as standard strain to validate *in vitro* stability of all the analyzed VNTRs. The selected isolate was grown on KB plates for 20 subsequent generations and for each of them genomic DNA was obtained by thermal denaturation: some bacteria, taken from 24 hours plate, were dissolved into molecular biology sterile water, mixed by vortexing and let incubate at 95°C for 10 minutes; then cells debris were pelleted by centrifugation (8.000 g for 3 minutes), DNA was retrieved as supernatant and stored at -20°C until use. The whole MLVA panel was applied to DNA from each generation and processed as described before to check any variation in repetition numbers of every single VNTRs.

Tab. 1. Characteristics of VNTRs initially selected: name of VNTR locus, TR sequence and length, sequences of forward and reverse primers and the corresponding annealing temperature. Sequences named as GMxxx come from Gironde & Manceau work (2012): TRs length and sequence in their paper were not reported, so these characteristics are known just for loci passing screening phase and so used in large scale analysis because of sequencing of amplicons.

VNTR	TR	TR length (bp)	Primers (5' → 3')	Annealing temperature (°C)
Psa-1	AGCTACA	7	F:CAAGCAGGAGATGGAAGAGC R:CATGCGGGCAATCTGATAGT	57
Psa-2	GGACTTGTCCGCGAAAAAGGCGGTGT ATTTCCCTGAAAATGAATCGTCTGAAC TAAGGGCTTCGCGGACAAGTCCGCTCC TACACAGTGCTGCTCTTGATTCTGGCC GAAGGCCGTGGGAAC	122	F:CCCCGACAATGCAAATAACT R:TATCCTCCGAAAATGCATCG	59
Psa-3	CTTCTAG	7	F:TTATCGGCGGGATGTGTATT R:ACTGCGTCTGGTCGATAACC	59
Psa-4	GCAGATTGGGCTTCAGCCCGGAGGGC GTGGCCA	33	F:ACGAGTCCGCTCCTACAAAA R:TACAACCAAGGTGGCCTGTT	59
Psa-5	AGAGGCA	7	F:GTAGGCCGCGCTTTCAAT R:TGCACTTCTTTTTCGCCTCT	59
Psa-6	CTATCGTTCCTCACGCTCCAGCCCAGG AATGCCTTGCGTGACGCTCTGCGTCAC AAATCTGCGCTTCGCTGCAGATTCAAG ATCGGACGCAGAGCGTCCAGAACGGC ATGCGACGCGGAGCGTCGCACGATAG TGAGGTTGTCGTTCTGCACGCTCCAGC CCAAACAT	169	F:CTGACAGTTTCGCAGTGACG R:TCAGGCGTTGAGGCTTATCT	59
Psa-7	GCGTCAAA	8	F:GCCTACCTTTTACGCCATGA R:CCGCCTCCAGTCAGGTTAAT	59
Psa-8	GGTGGTGTA	9	F:GTCATTGGCGAACTGATCCT R:CTTTTCATGCTGAAAGTCATGC	59
Psa-9	CCTGTGGGAGCGAGCTTGCTCGCGAAA GCGGAGTCACGTTGAGCATAAATCATC GGCAGCCATGCAGGCCTCTTCCTGAGC AAGCTCACTCCCACATCAGAGTACACA TGCC	112	F:CGCTGTCTGGCTTTGAAAAT R:TAGGACGGCCGAAGGTTTAT	61
Psa-10	TTTCGAT	7	F:AAGCCTGAGTAAGCGGTTCA R:GCCCCAGTCCCAGTTGTAAT	59
GM285	TGCACTCC	8	F:CGTGTCACTGAAGTCACCAT R:TATTTACCCGGTGTTGAGGC	54.8
GM1019	CCTGCA	6	F:CGAGTTCTATTTGCGTCAGG R:TGTCCAGCGTAATCTTGCTC	54.6
GM1409	/	/	F:CGGCAAGTATGCCTTGTGG R:AAGCCTGAGCATCTGGAATA	55.5
GM1613	GCTCAAA	7	F:CTGGCACGAGACGAGTCC R:GCTGAGCTTGAAGGAGACG	51.4
GM2222	/	/	F:ACGTGCATCGAAACCATCTG R:CCTTGGTGCCACGGTTTATA	57.8
GM2704	/	/	F:CGATGGGCAAACACGCAATC R:CAACAAGCCGTTGCATTCTC	59.8
GM2852	CAAGCTG	7	F:TGGGTGGAATACAGCCGCCA R:CTTGTTCGGGAGCGGCAAGCT	65.8
GM3097	/	/	F:CATGATGGTCGACTGCAAGG R:CCGCTGGCAATTTCATTGGG	61.3

Tab. 2. Whole set of Psa strains involved in MultiLocus VNTR Analysis. Those marked by † made up the initial screening group; the strains marked by * have been completely sequenced and are available as draft genomes on NCBI databank.

Name	Source	Year	Continent	Country	Region	Locality	Host species	Cultivar
PSA_7285	CFBP	2008	Europe	Italy	Veneto	Treviso	*A. chinensis*	Jin Tao
PSA_7286*†	CFBP	2008	Europe	Italy	Latium	Latina	*A. chinensis*	Hort-16A
PSA_7287	CFBP	2008	Europe	Italy	Latium	Latina	*A. deliciosa*	Hayward
PSA_829	UNITUS	2011	Europe	Spain	Galicia	Borguiera Tomino	*A. chinensis*	Jin Tao
PSA_830†	UNITUS	2011	Europe	Spain	Galicia	Borguiera Tomino	*A. chinensis*	Jin Tao
PSA_822	UNITUS	2011	Europe	Portugal	Viana do Castelo	Valença	*A. deliciosa*	Bo.Erica
PSA_832†	UNITUS	2011	Europe	Portugal	Aveiro	S.ta Maria da Feira	*A. deliciosa*	Hayward
PSA_834	UNITUS	2011	Europe	Portugal	Aveiro	S.ta Maria da Feira	*A. deliciosa*	Hayward
PSA_2F†	Anses	2010	Europe	France	Rhone Alpes	-	*A. deliciosa*	Hayward
PSA_3F	Anses	2010	Europe	France	Rhone Alpes	-	*A. deliciosa*	Hayward
PSA_9F	Anses	2011	Europe	France	Rhone Alpes	-	Actinidia sp.	Unknown
PSA_LSV38-71	-	2011	Europe	Switzerland	-	-	Actinidia sp.	Unknown
PSA_IT92*	CRA-FRU	1992	Europe	Italy	Latium	Latina	*A. deliciosa*	Hayward
PSA_2.34a	LTZ-Augustemberg	2013	Europe	Germany	Bavaria	-	*A. chinensis*	Red Sweetheart
PSA_2.34b	LTZ-Augustemberg	2013	Europe	Germany	Bavaria	-	*A. chinensis*	
PSA_TEIC801	T.E.I. Crete	2014	Europe	Greece	Central Macedonia	Mylotopos Pellas	*A. deliciosa*	Hayward
PSA_TEIC805	T.E.I. Crete	2014	Europe	Greece	Central Macedonia	Mylotopos Pellas	*A. deliciosa*	Hayward
PSA_TEIC806	T.E.I. Crete	2014	Europe	Greece	Central Macedonia	Mylotopos Pellas	*A. deliciosa*	Hayward
PSA_TEIC807	T.E.I. Crete	2014	Europe	Greece	Central Macedonia	Mylotopos Pellas	*A. deliciosa*	Hayward
PSA_TEIC819	T.E.I. Crete	2014	Europe	Greece	Central Macedonia	Mylotopos Pellas	*A. deliciosa*	Hayward
PSA_TEIC823	T.E.I. Crete	2014	Europe	Greece	Central Macedonia	Mylotopos Pellas	*A. deliciosa*	Hayward
PSA_TEIC824	T.E.I. Crete	2014	Europe	Greece	Central Macedonia	Mylotopos Pellas	*A. deliciosa*	Hayward
PSA_TEIC826	T.E.I. Crete	2014	Europe	Greece	Central Macedonia	Mylotopos Pellas	*A. deliciosa*	Hayward
PSA_K2T†	Bastas K.K.	2011	Asia	Turkey	Rize	-	*A. deliciosa*	Hayward
PSA_18804*	ICMP	2010	Australasia	New Zealand	Bay of Plenty	Te Puke	*A. chinensis*	Hort 16A
PSA_18882	ICMP	2010	Australasia	New Zealand	Nelson	Motueka	*A. chinensis*	Hort 16A
PSA_19497	ICMP	2010	Australasia	New Zealand	Bay of Plenty	Te Puke	*A. chinensis*	Hort 16A
PSA_19441	ICMP	2012	Australasia	Australia	Victoria	-	*A. chinensis*	Unknown

PSA_19486	ICMP	2013	Australasia	Australia	Western Australia	-	*A. chinensis*	Unknown
PSA_19440	ICMP	2011	Australasia	Australia	Victoria	-	*A. chinensis*	Unknown
PSA_18839	ICMP	2011	Australasia	New Zealand	Bay of Plenty	Te Puke	*A. deliciosa*	Hayward
PSA_18875†	ICMP	2011	Australasia	New Zealand	Bay of Plenty	Te Puke	*A. deliciosa*	Hayward
PSA_19200	ICMP	2011	Australasia	New Zealand	Auckland	-	Actinidia sp.	Unknown
PSA_23663	KCTC	1989	Asia	Korea	Jeollanam-do	Naju-si	*A. chinensis*	Unknown
PSA_23664†	KCTC	1989	Asia	Korea	Jeollanam-do	Naju-si	*A. chinensis*	Unknown
PSA_23665	KCTC	1989	Asia	Korea	Jeollanam-do	Naju-si	*A. chinensis*	Unknown
PSA_K2†	Koh Y.J.	1997	Asia	Korea	-	-	*A. chinensis*	Unknown
PSA_K3	Koh Y.J	1999	Asia	Korea	Jeju-do	Bukjeju-gun	*A. deliciosa*	Hayward
PSA_K4	Koh Y.J	1999	Asia	Korea	Jeollanam-do	Wando-gun	*A. deliciosa*	Hayward
PSA_K5	Koh Y.J	2008	Asia	Korea	Jeju-do	Nabeup-ri	*A. deliciosa*	Hayward
PSA_K6	Koh Y.J	2010	Asia	Korea	Jeju-do	Tosan-ri	*A. chinensis*	Hort-16A
PSA_K7	Koh Y.J	2008	Asia	Korea	Jeju-do	Tosan-ri	*A. chinensis*	Hort-16A
PSA_K8	Koh Y.J	2008	Asia	Korea	Jeju-do	Sangmo-ri	*A. chinensis*	Hort-16A
PSA_K9	Koh Y.J	2008	Asia	Korea	Jeju-do	Odeung-dong	*A. chinensis*	Hort-16A
PSA_K10	Koh Y.J	1999	Asia	Korea	Jeollanam-do	Jindo-gun	*A. deliciosa*	Hayward
PSA_K11	Koh Y.J	2008	Asia	Korea	Jeollanam-do	Wando-gun	*A. deliciosa*	Hayward
PSA_K12	Koh Y.J	2009	Asia	Korea	Jeju-do	Tosan-ri	*A. chinensis*	Hort-16A
PSA_131WD	Koh Y.J.	2013	Asia	Korea	Jeollanam-do	Wando-eup	*A. chinensis*	Hong Yang
PSA_132WD	Koh Y.J.	2013	Asia	Korea	Jeollanam-do	Wando-eup	*A. chinensis*	Hong Yang
PSA_133WD	Koh Y.J.	2013	Asia	Korea	Jeollanam-do	Wando-eup	*A. chinensis*	unknown new variety
PSA_134WD	Koh Y.J.	2013	Asia	Korea	Jeollanam-do	Wando-eup	*A. chinensis*	unknown new variety
PSA_134KBS	Koh Y.J.	2013	Asia	Korea	Jeollanam-do	Yeongam-eup	*A. deliciosa*	Hayward
PSA_SYS1	Koh Y.J.	2011	Asia	Korea	Jeollanam-do	Goheung-gun	*A. chinensis*	Unknown
PSA_SYS2	Koh Y.J.	2011	Asia	Korea	Jeollanam-do	Goheung-gun	*A. chinensis*	Unknown
PSA_SYS4	Koh Y.J.	2011	Asia	Korea	Jeollanam-do	Goheung-gun	*A. chinensis*	Unknown
PSA_KW1*	Takikawa Y.	1984	Asia	Japan	Chūbu	Shizuoka	*A. deliciosa*	Hayward
PSA_KW11*	Takikawa Y.	1984	Asia	Japan	Chūbu	Shizuoka	*A. deliciosa*	Hayward
PSA_KW30	CFBP	1984	Asia	Japan	Chūbu	Shizuoka	*A. deliciosa*	Hayward
PSA_KW41*	CFBP	1984	Asia	Japan	Chūbu	Shizuoka	*A. deliciosa*	Hayward
PSA_PA429	CFBP	1987	Asia	Japan	-	-	*A. chinensis*	Unknown
PSA_PA459*	CFBP	1988	Asia	Japan	-	-	*A. chinensis*	Unknown
PSA_2818	Takikawa Y.	2011	Asia	Japan	Chūbu	Shizuoka	*A. chinensis*	Rainbow red
PSA_2819	Takikawa Y.	2011	Asia	Japan	Chūbu	Shizuoka	*A. chinensis*	Rainbow red
PSA_2820	Takikawa Y.	2011	Asia	Japan	Shikoku	Ehime	*A. deliciosa*	Hayward
PSA_2726	Takikawa Y.	2009	Asia	Japan	Chūbu	Shizuoka	*A. deliciosa*	Unknown
PSA_CH2010-5	Huang L.L.	2010	Asia	China	Shaanxi	Sheng (Dangdong)	*A. chinensis*	Hong Yang
PSA_CH2010-6*†	Huang L.L.	2010	Asia	China	Shaanxi	Sheng (Dangdong)	*A. chinensis*	Hong Yang
PSA_CH2010-7	Huang L.L.	2010	Asia	China	Shaanxi	Sheng (Dangdong)	*A. chinensis*	Hong Yang
PSA_M218	Zhao Z.B.	2010	Asia	China	Shaanxi	Yangling (Xianyang)	*A. deliciosa*	Xi Xuan
PSA_M228	Zhao Z.B.	2010	Asia	China	Shaanxi	Baoji (Mei County)	*A. chinensis*	Hong Yang
PSA_M23	Zhao Z.B.	2010	Asia	China	Shaanxi	Baoji (Mei County)	*A. chinensis*	Hong Yang
PSA_M122	Zhao Z.B.	2010	Asia	China	Shaanxi	Baoji (Mei County)	*A. chinensis*	Hong Yang
PSA_JILO4†	UNITUS	2012	Asia	China	Anhui	Yuexi	*A. chinensis*	JinFeng
PSA_JILO8	UNITUS	2012	Asia	China	Anhui	Yuexi	*A. chinensis*	JinFeng
PSA_JILO16	UNITUS	2012	Asia	China	Anhui	Yuexi	*A. chinensis*	JinFeng
PSA_JILO17	UNITUS	2012	Asia	China	Anhui	Yuexi	*A. chinensis*	JinFeng

PSA_JILO21	UNITUS	2012	Asia	China	Anhui	Yuexi	*A. chinensis*	JinFeng
PSA_JILO22	UNITUS	2012	Asia	China	Anhui	Yuexi	*A. chinensis*	JinFeng
PSA_JILO23	UNITUS	2012	Asia	China	Anhui	Yuexi	*A. chinensis*	JinFeng
PSA_JILO24	UNITUS	2012	Asia	China	Anhui	Yuexi	*A. chinensis*	JinFeng
PSA_JILO26	UNITUS	2012	Asia	China	Anhui	Yuexi	*A. chinensis*	JinFeng
PSA_JILO27	UNITUS	2012	Asia	China	Anhui	Yuexi	*A. chinensis*	JinFeng
PSA_JILO30	UNITUS	2012	Asia	China	Anhui	Yuexi	*A. chinensis*	JinFeng
PSA_LOLO1	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO3	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO4	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO5	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO6	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO12	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO13	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO14	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO15	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO16	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO17	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO18	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO20	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO21	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO26	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO27	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO28	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO29	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO31	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO32	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO37	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO38	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO39	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO40	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO41	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_LOLO42	UNITUS	2012	Asia	China	Guizhou	Xiu Wen	*A. deliciosa*	Guichang
PSA_INS	UNITUS	2012	Asia	China	Anhui	Yuexi	Insect (C*icadellidae*)	
PSA_HAXA1	UNITUS	2012	Asia	China	Shaanxi	Xi'an	*A. deliciosa*	Hayward
PSA_HAXA2	UNITUS	2012	Asia	China	Shaanxi	Xi'an	*A. deliciosa*	Hayward
PSA_HAXA3	UNITUS	2012	Asia	China	Shaanxi	Xi'an	*A. deliciosa*	Hayward
PSA_HAXA4	UNITUS	2012	Asia	China	Shaanxi	Xi'an	*A. deliciosa*	Hayward
PSA_HAXA5	UNITUS	2012	Asia	China	Shaanxi	Xi'an	*A. deliciosa*	Hayward
PSA_HAXA6	UNITUS	2012	Asia	China	Shaanxi	Xi'an	*A. deliciosa*	Hayward
PSA_HAXA7	UNITUS	2012	Asia	China	Shaanxi	Xi'an	*A. deliciosa*	Hayward
PSA_HAXA8	UNITUS	2012	Asia	China	Shaanxi	Xi'an	*A. deliciosa*	Hayward
PSA_HYM1	UNITUS	2012	Asia	China	Sichuan	Mian Yang	*A. chinensis*	Hong Yang
PSA_HYM2	UNITUS	2012	Asia	China	Sichuan	Mian Yang	*A. chinensis*	Hong Yang
PSA_HYM3	UNITUS	2012	Asia	China	Sichuan	Mian Yang	*A. chinensis*	Hong Yang
PSA_HYM4	UNITUS	2012	Asia	China	Sichuan	Mian Yang	*A. chinensis*	Hong Yang
PSA_MC1	Zhu L.W.	2013	Asia	China	Anhui	Jinzhai	*Setaira viridis (L.) Beauv.*	-
PSA_MC2	Zhu L.W.	2013	Asia	China	Anhui	Jinzhai	*Setaira viridis (L.) Beauv.*	-
PSA_HWD3	Zhu L.W.	2012	Asia	China	Shaanxi	Xi'an	*A. deliciosa*	-
PSA_HWD4	Zhu L.W.	2012	Asia	China	Shaanxi	Xi'an	*A. deliciosa*	-
PSA_HWD5	Zhu L.W.	2012	Asia	China	Shaanxi	Xi'an	*A. deliciosa*	-
PSA_AHPP5	Zhu L.W.	2013	Asia	China	Anhui	Yuexi	*Philagra quadrimaculata Schmidt*	-

PSA_AHPP7	Zhu L.W.	2013	Asia	China	Anhui	Yuexi	*Philagra quadrimaculata Schmidt*	-
PSA_GMC3	Zhu L.W.	2013	Asia	China	Anhui	Jinzhai	*Alternanthera philoxeroides (Mart.) Griseb.*	-
PSA_GMC2	Zhu L.W.	2013	Asia	China	Anhui	Jinzhai	*Alternanthera philoxeroides (Mart.) Griseb.*	-
PSA_JZHY7	Zhu L.W.	2013	Asia	China	Anhui	Jinzhai	*A. deliciosa*	-
PSA_JZZM11	Zhu L.W.	2013	Asia	China	Anhui	Jinzhai	*A. deliciosa*	-
PSA_WT1	Zhu L.W.	2013	Asia	China	Anhui	Yuexi	*Pauwlonia fortunei (Seem.) Hemsl.*	-
PSA_SH5	Zhu L.W.	2013	Asia	China	Shanghai	Jiading	*A. deliciosa*	-
PSA_WT2	Zhu L.W.	2013	Asia	China	Anhui	Yuexi	*Pauwlonia fortunei (Seem.) Hemsl*	-
PSA_SCHY24	Zhu L.W.	2013	Asia	China	Sichuan	Pengzhou	*A. deliciosa*	-
PSA_SCHY25	Zhu L.W.	2013	Asia	China	Sichuan	Pengzhou	*A. deliciosa*	-
PSA_SH28	Zhu L.W.	2013	Asia	China	Shanghai	Jiading	*A. deliciosa*	-
PSA_19439*	ICMP	2011	South America	Chile	VII Region of Maule	-	*A. deliciosa*	unknown new variety
PSA_19457	ICMP	2010	South America	Chile	VII Region of Maule	-	*A. deliciosa*	unknown variety
PSA_19438	ICMP	2011	South America	Chile	VII Region of Maule	-	*A. deliciosa*	unknown new variety
PSA_19456	ICMP	2010	South America	Chile	VII Region of Maule	-	*A. chinensis*	unknown variety
PSA_1_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	San Javier	*A. deliciosa*	Hayward
PSA_2_Chile	SAG-Chile	2013	South America	Chile	Bio Bio	San Carlos	*A. deliciosa*	Hayward
PSA_3_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	Longavi	*A. deliciosa*	Hayward
PSA_4_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	Retiro	*A. deliciosa*	Hayward
PSA_5_Chile	SAG-Chile	2013	South America	Chile	Bio Bio	San Carlos	*A. deliciosa*	Hayward
PSA_6_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	Talca	*A. deliciosa*	Hayward
PSA_7_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	Longavi	*A. chinensis*	Kis Y374
PSA_8_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	Retiro	*A. deliciosa*	Hayward

PSA_9_Chile	SAG-Chile	2013	South America	Chile	Bio Bio	San Ignacio	*A. deliciosa*	Hayward
PSA_10_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	Vila Alegre	*A. deliciosa*	Hayward
PSA_11_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	San Javier	*A. deliciosa*	Hayward
PSA_12_Chile	SAG-Chile	2013	South America	Chile	Bio Bio	Bulnes	*A. deliciosa*	Hayward
PSA_13_Chile	SAG-Chile	2014	South America	Chile	VII Region of Maule	Molina	*A. deliciosa*	Matua
PSA_14_Chile	SAG-Chile	2013	South America	Chile	Bio Bio	San Ignacio	*A. deliciosa*	Hayward
PSA_15_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	Retiro	*A. deliciosa*	Hayward
PSA_16_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	Colbun	*A. deliciosa*	Hayward
PSA_17_Chile	SAG-Chile	2013	South America	Chile	Bio Bio	San Ignacio	*A. deliciosa*	Hayward
PSA_18_Chile	SAG-Chile	2013	South America	Chile	Bio Bio	Bulnes	*A. deliciosa*	Hayward
PSA_19_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	Vila Alegre	*A. deliciosa*	Hayward
PSA_20_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	Retiro	*A. deliciosa*	Hayward
PSA_21_Chile	SAG-Chile	2013	South America	Chile	Bio Bio	Bulnes	*A. deliciosa*	Hayward
PSA_22_Chile	SAG-Chile	2013	South America	Chile	Bio Bio	San Ignacio	*A. deliciosa*	Hayward
PSA_23_Chile	SAG-Chile	2014	South America	Chile	VII Region of Maule	Molina	*A. deliciosa*	Matua
PSA_24_Chile	SAG-Chile	2013	South America	Chile	VII Region of Maule	Yerbas Buenas	*A. deliciosa*	Hayward
PSA_25_Chile	SAG-Chile	2013	South America	Chile	Bio Bio	San Carlos	*A. deliciosa*	Hayward

5. RESULTS

5.1 *In silico* search of candidate VNTRs

Each of the three genomes analyzed by TRDB gave one set of Tandem Repeats (TRs): in particular CFBP7286 turns out to have 157 TRs, CH2010-6 186 and M302091 104. These groups were then compared two by two by Position Comparison tool and primers were designed where possible, discarding TRs with lacking or too short sequenced flanking regions.
The main features of VNTR loci passing the screening phase, such as position on WGS sequence of the strain M302091 (NCBI WGS Project AEAL01) and putative function of open reading frames (ORFs) interested by VNTR amplicons are listed in table 3.

5.2 PCR and capillary electrophoresis

In the screening phase, all the VNTRs loci tested were properly amplified and produced a single clear amplicon of identical dimensions in reiterated experiments. The first screening of the whole panel of 18 VNTRs on 10 representative strains has led to the selection of 12 polymorphic VNTRs (Table 4) with adequate discriminatory power(Fig. 6), and to the exclusion of 6 loci (2 loci on 10 from our selection, and 4 on 8 from the panel from Gironde & Manceau) that have always produced indifferently the same amplicons among all the tested Psa strains.
The panel of the remaining 12 VNTRs (8 from this study and 4 from GM set) was then used to amplify 168 Psa in total whose identity was previously confirmed by other molecular assays (data not shown); some VNTRs showed a discrete discriminatory power even if considered singularly (Fig. 7), whilst others described a narrow number of allelic variation. 10 on 12 VNTR loci were successfully amplified with all the strains, whilst the locus Psa-9 failed to amplify all the 6 strains belonging to low virulent strains from New Zealand and Australia, and the 5 strains isolated in South Korea during 2013 in Wando-eup area (PSA_131WD-PSA_134KBS), and the locus GM285 failed to amplify again all the 6 strains belonging to Psa-4 haplotype. The missing amplification was coded as -1 in the matrix of repeat numbers to be used for the subsequent computational analysis.

In some cases, the number of TRs obtained from capillary analysis didn't fit correctly with data obtained from those strains whose WGS sequences are deposited in NCBI; each ambiguous datum was definitely resolved by sequencing the corresponding amplicon and counting manually the entire and truncated repetitions (data not shown).

Only when the data for all the VNTR loci and all the 168 Psa strains were definitely validated by double repetition of the whole process (PCR and capillary electrophoresis), and by sequencing of amplicons of all the VNTR loci for 3 representative strains and of those loci in which the number of TRs resulted out of line with WGS data, any further elaboration was performed. Sequencing allowed also to find out the particular structure of Psa-7 locus: it is made up of a polymorphic repeated unit followed by a monomorphic region and again the same polymorphic repeated sequence. Within statistical elaboration, every TR was considered singularly as a VNTR locus, named Psa-7A and Psa-7B, leading the MLVA panel from 12 to 13 loci. Details for each VNTR locus about amplicons dimensions, length of the flanking regions, number of repeats, truncated repeats are reported in table 4.

5.3 General characteristics of MLVA panel

Among 168 Psa strains, 72 haplotypes were overall detected pooling the data from 13 VNTRs, and the number of alleles per locus varied from 3 (GM2852) to 13 (GM1019). The correlation between number of alleles and length of TR was evaluated (Fig. 8). The HGDI and Simpson's diversity indexes were assessed for each VNTR locus and ranged respectively from 0,427 to 0,855, and from 0,423 to 0,847; the complete diversity indices results are reported in table 5.

Additional information of key significance for a correct interpretation of VNTR data were achieved by direct sequencing of a selection of VNTRs amplicons. In particular, apart the amplification failure of 2 on 12 VNTR loci, other anomalies were detected in Psa strains referable to the low virulent group (haplotype Psa-4): the VNTR Psa-1 usually has a right flanking region of 80 nucleotides, but the corresponding sequence of all the 6 low virulent strains tested lacks in 7 nucleotides at position 32-38 after the end of TRs, so that for the calculation of exact number of TRs for strains belonging to low virulent haplotype, 7 base pairs have to be previously detracted from the sum of flanking regions (136 bp instead of 143); similarly, in VNTR Psa-3, the left

flanking region of low virulent strains misses of 2 nucleotides in position 74-75 from the beginning of the amplicon; again, one additional nucleotide is retrievable in right flanking region of VNTR Psa-4 of low virulent strains. Furthermore, several single nucleotide polymorphisms can be detected in flanking regions of the same low virulent strains.
However, also in few other Psa strains discrepancies were found: the left flanking region of VNTR GM285 have different lengths according to the geographic origin of the strains: in fact, European, Chinese, Chilean and New Zealander virulent strains have a 133 bp left flanking region, whilst the correspondent region in Japanese and Korean strains have 125 bp.

5.4 Elaboration by "diversity panel"

Bionumerics elaboration was firstly carried out on the whole set of 168 Psa isolates considering only the 6 loci showing a haplotypes number equal to or up to 5 plus Psa-1 (6 haplotypes) inserted in the "diversity panel" (Psa-1, Psa-4, Psa-7B, Psa-9, GM1613, GM2852; Tab. 4 marked by *). Clonal complex (CC) was defined as similarity group including strains displaying not more than 2 differences on 6 loci of the MLVA profile or in other words dissimilar for less than approximately 33%.
UPGMA dendrogram and MST were generated and it was noticed that they were perfectly adaptable and consistent between them and in respect to previously determined division into 4 populations or haplotypes, obtained mostly from more expensive and time-consuming techniques such as genome-wide SNPs analysis (McCann, 2013) (Fig. 9) and MLSA (Chapman, 2012) (Fig. 10).
Overall categorical MST analysis provided 9 clonal complexes and 2 singletons (Fig. 10).

5.5 Elaboration by whole MLVA panel

The whole panel of 13 VNTR loci was applied to the entire collection of Psa to perform even in this case a categorical MST; however, because of the more congruous number of loci, cut-off value for determining clonal complexes was set at 3 differences on 13, corresponding to a percentage of dissimilarity of about 23%.

11 clonal complexes and 5 singletons resulted from the elaboration (Fig. 11).

The 3 macrogroups (Japan-Korea, China and Hypervirulent) were analyzed separately by applying the 13 loci MLVA panel to better elucidate subtle differences within every single group, with the exception of Psa-LV from Australia and New Zealand excluded from the subsequent elaboration as they shared only 2 loci on 13 with the nearest "China CC E".

Japan and South Korea strains were analyzed together because of several interconnections between them: overall they were comprised into 5 CCs and 4 singletons (Fig. 12): "Japan CC A" and "Japan CC C" constituted exclusively by Japanese strains; "Japan-Korea CC B" grouping Korean, Japanese and the Italian strain of 1992 PSA_IT92; "Korea CC D" composed by most of the Korean strains and the Turkish PSA_K2T; "Korea CC E" made of Korean strains.

"Japan CC A" is formed by PSA_2818 and PSA_2819, both isolated in 2011 in Chūbu region (Shizuoka), whilst the remaining 4 strains from the same site but dated back to 1984 (PSA_KW1, PSA_KW11, PSA_KW30 and PSA_KW41) were included in "Japan-Korea CC B", together with PSA_IT92 (Italy, 1992), PSA_23663 and PSA_23664, both from South Korea (Jeollanam, Naju-si) isolated in 1989. The two Japanese strains PSA_PA429 and PSA_459, isolated respectively in 1988 and 1989 from unknown locality, formed the "Japan CC C". "Korea CC D" encompassed the most of the analyzed Korean strains characterized by various site and date of isolation: PSA_K3 (1993), PSA_K5 (2008), PSA_K6 (2010), PSA_K7 (2008), PSA_K8 (2008), PSA_K9 (2008), PSA_K12 (2009) coming from Jeju region but from different localities; PSA_K4 (1999), PSA_K10 (1999) PSA_K11 (2008), PSA_131WD (2013), PSA_132WD (2013), PSA_133WD (2013), PSA_134WD (2013) and PSA_134KBS (2013) all isolated from several zones of Jeollanam region. Moreover in the "Korea CC D" also the unique Turkish strain of the study was found out (PSA_K2T, Rize, 2011), suggesting an intercontinental contagion event. The last clonal complex, "Korea CC E" was made up of PSA_SYS1, PSA_SYS2 and PSA_SYS4, homogenous for year and site of isolation (Jeollanam, Goheung-gun, 2011). Japan-Korea group encompasses the greatest number of singletons, two from Korea and two from Japan, in respect to China and Hypervirulent ones: PSA_23665 (Korea, Jeollanam, Naju-si, 1989), PSA_2820 (Japan, Shikoku, Ehime, 2011), PSA_2726 (Japan, Chūbu, Shizuoka, 2009), PSA_K2 (Korea, unknown location, 1997).

China group resulted to be the most variegated and it was described by 6 clonal complexes, generally corresponding to geographic region of provenience (Fig. 13).

"China CC A" included all the strains isolated in 2012 in Guizhou region (Guiyang, district of Xiuwen): PSA_LOLO1, PSA_LOLO3, PSA_LOLO4, PSA_LOLO5, PSA_LOLO6, PSA_LOLO12, PSA_LOLO13, PSA_LOLO14, PSA_LOLO15, PSA_LOLO16, PSA_LOLO17, PSA_LOLO18, PSA_LOLO20, PSA_LOLO21, PSA_LOLO26, PSA_LOLO27, PSA_LOLO28, PSA_LOLO29, PSA_LOLO31, PSA_LOLO32, PSA_LOLO37, PSA_LOLO38, PSA_LOLO39, PSA_LOLO40, PSA_LOLO41, PSA_LOLO42). Moreover, PSA_HYM1 (Sichuan, Deyang, district of Mianzhu) and PSA_HAXA4 (Shaanxi, Xi'an, district of Huxian) both isolated in 2012 were found in this clonal complex. "China CC B" consisted of 5 strains coming from different locality in Shaanxi region in 2010: PSA_CH2010-5, PSA_CH2010-6, PSA_CH2010-7 (Sheng, district of Dangdong); PSA_M23, PSA_M122 (Baoji, district of Mei). In the initial 13 loci-elaboration on the whole set of Psa (Fig. 11) the "China CC B" was included in the same clonal complex of the Hypervirulent group, making suppose that Shaanxi province could be the start point of 2008 lethal outbreak. "China CC C" resulted to be formed by only 2 strains, PSA_SCHY24 and PSA_SCHY25, both isolated in Pengzhou in Sichuan region in 2013. All the strains belonging to "China CC D" were isolated in Jinzhai in the Anhui region in 2013, but the peculiarity is that they resided on several hosts, two of which different from *Actinidia* spp.: PSA_GMC3, PSA_GMC2 were isolated from *Alternanthera philoxeroides*, PSA_MC1 and PSA_MC2 from *Setaira viridis* and PSA_JZZM11 from *Actinidia deliciosa*. "China CC E" was a very heterogeneous group concerning especially area of isolation and hosts, including: all the strains of 2012 hailing from Anqing, district of Yuexi, Anhui region (PSA_JILO4, PSA_JILO8, PSA_JILO16, PSA_JILO17, PSA_JILO21, PSA_JILO22, PSA_JILO23, PSA_JILO24, PSA_JILO26, PSA_JILO27, PSA_JILO30); PSA_HAXA1, PSA_HAXA2, PSA_HAXA3, PSA_HAXA5, PSA_HAXA6, PSA_HAXA7, PSA_HAXA8 from Shaanxi region (Xi'an, district of Huxian) in 2012; PSA_HYM2, PSA_HYM3, PSA_HYM4 from Sichuan region (Deyang, Mianzhu) in 2012; PSA_INS isolated in 2012 from an insect belonging to *Cicadellidae* found in Anhui region (Anqing, district of Yuexi); PSA_AHPP5 and PSA_AHPP7 isolated in 2013 from *Philagra quadrimaculata* in Anhui region (Anqing, district of Yuexi); PSA_HWD3, PSA_HWD4, PSA_HWD5 isolated in 2012 from Xi'an in Shaanxi region; PSA_WT1 and PSA_WT2 found on *Pauwlonia fortunei* in 2013 in Anhui region (Anqing, district of Yuexi); PSA_SH5 and PSA_SH28 isolated from Jiading in Shangai region in 2013. Furthermore, "China CC E" is the group most similar to the unique Hypervirulent singleton PSA_2.34b from Germany, with whom it shared 9

loci on 13. “China CC F” encompassed PSA_JZHY7, coming from Anhui region (Jinzhai) in 2013, PSA_M218 from Xianyang district of Yangling, and PSA_M228 from Baoji, district of Mei, both in Shaanxi region and isolated in 2010.

Hypervirulent group, composed of all the strains collected starting from 2008 outbreak in Europe, Chile and New Zealand V demonstrated to be the most homogeneous group, forming a unique clonal complex (“Hyper CC”) and revealing just 1 singleton (PSA_2.34b from Germany), actually more similar to “China CC E” than to European strains, as mentioned before (Fig. 14). Interestingly, in the “Hyper CC” also located 5 Chinese strains dating back to 2010 and isolated in Shaanxi province,corresponding to “China CC B” (PSA_M122, PSA_M23, PSA_CH2010-5, PSA_CH2010-6, PSA_CH2010-7) and so suggesting a Chinese origin for today’s pandemy.

UPGMA analysis, run on the same three macrogroups Japan-Korea, China and Hypervirulent, provided results well suited to those obtained from MST elaboration, confirming the reliability of the applied MLVA panel: in general each clonal complex perfectly corresponded to one or encompassed more than one similarity cluster. Overall 24 similarity clusters and 6 singletons were detected.

Japan-Korea group was divided into 6 clusters, numbered from 1 to 6 (Fig. 15). Cluster 1, constituted by all the Korean strains from 1999 to 2010 plus the Turkish strain, and cluster 2, containing exclusively Korean strains of 2013, were included into “Korea CC D”; cluster 3 perfectly coincided to “Korea CC E”, whilst cluster 4, 5 and 6 corresponded respectively to “Japan-Korea CC B”, “Japan CC A” and “Japan CC C”; out of any similarity group, there were all the 4 singletons, already identified in MST analysis.

Concerning the China group, 12 similarity clusters were found, indicated from 7 to 18 (Fig. 16). Cluster 12, cluster 16, cluster 17 and cluster 18 were perfectly suited to respectively “China CC F”, “China CC B”, “China CC D” and “China CC C”. “China CC E” resulted to encompass clusters from 7 to 11, which in general reflected geographic distribution of Psa strains; the last clonal complex, “China CC A” was composed by clusters 13, 14 and 15, all the 3 constituted by strains found in Guizhou province with just two exceptions in cluster 14 (PSA_HAXA4 from Shaanxi and PSA_HYM1 from Sichuan), what’s more datum confirmed by MST results.

The unique “Hyper CC” was partitioned into 6 clusters, from 19 to 24 (Fig. 17). Cluster 19 was formed by exclusively Chilean strains, however some of them were present in additional 2 clusters, 21 and 22, together with European strains; moreover the Chilean strain PSA_CHILE10 came out as

a singleton within UPGMA elaboration. Cluster 20 collected the 3 New Zealander V strains; cluster 23 was composed by only 2 European strains, one French and the other Portuguese, whilst cluster 24 encompassed Chinese strains from Shaanxi province isolated in 2010. The only singleton of Hypervirulent group, the German PSA_2.34b, even in this case was again confirmed as such.

5.6 *In vitro* stability test

By plating for 20 consecutive generations the reference strain PSA_7286 and by applying the entire MLVA panel to each, tendency to change repetition number of every single VNTR was evaluated: overall stability resulted to be very remarkable since in any case no differences were observed and for each locus a band with the same length along the 20 generations was always produced.

Tab. 3. Position and putative function of ORFs contained into VNTRs, involved in large scale analysis. Reference *in silico* strain is Japanese M302091.

VNTR	Position on M302091	ORFs position	ORFs putative function
Psa-1	Contig 63.4 1649-1815	1649-1678	end of "phosphoadenosine phosphosulfate reductase" gene
		1679-1763	Unknown
		1764-1815	beginning of "transcriptional regulator CysB" gene
Psa-3	Contig 21.9 1311-1476	1311-1374	end of "dipeptide transporter ATP-binding subunit" gene
		1375-1454	Unknown
		1455-1476	beginning of "uncharacterized iron-regulated membrane protein" gene
Psa-4	Contig 3.7 8452-8557	8452-8557	unknown
Psa-5	Contig 40.2 22258-22439	22258-22332	end of "hypothetical protein" gene
		22333-22439	Unknown
Psa-7	Contig 41.2 1961-2152	1961-2152	Unknown
Psa-8	Contig 1.13 8812-8993	8812-8835	end of "leucine-rich repeat domain-containing protein" gene
		8836-8993	Unknown
Psa-9	Contig 3.3 8172-8187	8172- 8187	end of "hypothetical protein" gene
	not found	-	-
	Contig 3.4 1-26	1-26	Unknown
Psa-10	Contig 46.2 19700-19953	19700-19708	end of "amino acid ABC transporter periplasmic aminoacid-binding protein" gene
		19709-19929	Unknown
		19930-19953	beginning of "amino acid ABC transporter permease" gene
GM285	Contig 60.2 2-192	2-192	Unknown
	not found	-	-
	Contig 60.1 1761-1900	1761-1900	Unknown
GM1019	Contig 89.2 6241-6368	6241-6368	end of "tellurite resistance protein TerA" gene
	not found	-	-
GM1613	Contig 63.4 1649-1815	1649-1678	end of "phospohoadenosine phosphosulfate reductase" gene
		1679-1763	Unknown
		1764-1815	beginning of "transcriptional regulator CysB" gene
GM2852	Contig 53.5 1051-1248	1051-1185	Unknown
		1186-1248	beginning of "glutamine amidotransferase, class-II protein" gene

Tab. 4. Characteristics of VNTRs used to perform large scale study. a,b,c) flanking length in LV isolates; d) length range of single repeated unit; e) locus not amplified in LV isolates; f) left flanking is 125 bp in Korean and Japanese strains and 133 bp for all the others; g) locus not amplified in LV neither Korean isolates of 2013. VNTRs marked by * are those constituting the "diversity panel".

VNTR	Left flanking (bp)	Right flanking (bp)	TR length (bp)	Numbers of alleles (entire TRs)	Final partial TR (bp)	Haplotypes number	Amplicons range (bp)
		THIS STUDY					
Psa-1*	63	80 (73[a])	7	3-9	3	6	153-202
Psa-3	86 (84[b])	62	7	3-13	4	10	164-236
Psa-4*	66	58 (59[c])	33	1-16	-	4	158-652
Psa-5	86	64	7	0-3	4	4	161-203
Psa-7A	68	91	8	0-6	-	6	175-207
Psa-7B*				0-4		4	
Psa-8	72	72	9	2-10	-	8	162-243
Psa-9*	26	31	99-112[d]	0-7	32	4	305-723[e]
Psa-10	79	70	7	2-17	6	12	162-260
		GIRONDE & MANCEAU					
GM285	125-133[f]	223	8	0-12	3	8	367-423[g]
GM1019	118	54	6	6-18	4	13	179-284
GM1613*	139	26	7	3-8	6	5	185-220
GM2852*	28	150	7	2-4	6	3	191-205

Tab. 5 Simpson's and Hunter-Gaston's indices of diversity calculated for each of VNTR. Loci are reported in decreasing order of index value, thus from the most to the less variable.

Simpson's diversity index				
Locus	***Diversity index***	***Confidence range***	***K***	***max(pi)***
GM1019	0.847	0.818 - 0.877	12	0.239
Psa-10	0.820	0.785 - 0.855	10	0.283
Psa-3	0.785	0.738 - 0.832	9	0.372
Psa-1	0.783	0.753 - 0.812	6	0.301
Psa-8	0.778	0.736 - 0.821	8	0.327
GM1613	0.756	0.726 - 0.787	5	0.345
Psa-5	0.755	0.723 - 0.787	7	0.301
Psa-7	0.685	0.643 - 0.727	5	0.434
GM285	0.679	0.601 - 0.758	8	0.522
Psa-9	0.570	0.515 - 0.626	4	0.549
Psa-4	0.451	0.352 - 0.550	4	0.717
GM2852	0.423	0.326 - 0.519	3	0.735
Hunter-Gaston's diversity index				
Locus	***Diversity index***	***Confidence range***	***K***	***max(pi)***
GM1019	0.855	0.825 - 0.884	12	0.239
Psa-10	0.827	0.792 - 0.862	10	0.283
Psa-3	0.792	0.745 - 0.839	9	0.372
Psa-1	0.790	0.760 - 0.819	6	0.301
Psa-8	0.785	0.743 - 0.828	8	0.327
GM1613	0.763	0.733 - 0.793	5	0.345
Psa-5	0.762	0.730 - 0.793	7	0.301
Psa-7	0.691	0.649 - 0.734	5	0.434
GM285	0.686	0.607 - 0.764	8	0.522
Psa-9	0.575	0.520 - 0.631	4	0.549
Psa-4	0.455	0.356 - 0.554	4	0.717
GM2852	0.427	0.330 - 0.523	3	0.735

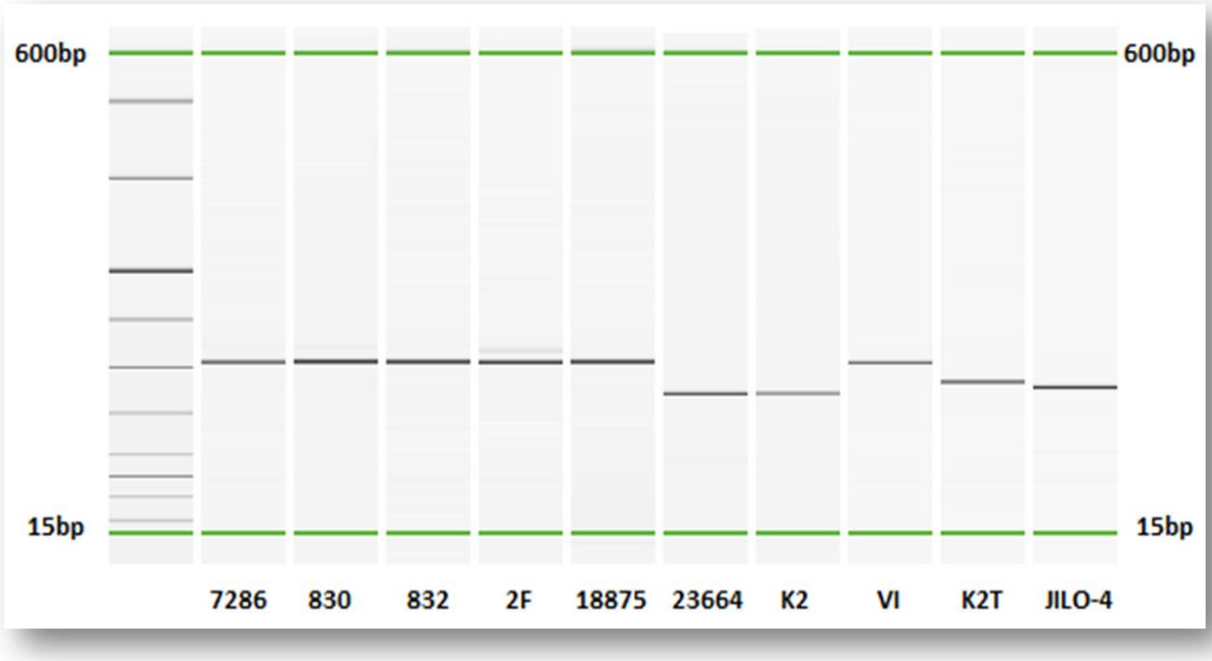


Fig. 6 Psa-1 on screening strains run by OM700 method of QIAxcel with 15-600 bp alignment marker. Lanes 1-11: 1) size marker 25-500 bp; 2) PSA_7286 (Italy, 2008): repetitions number 9; 3) PSA_830 (Spain, 2011): repetitions number 9; 4) PSA_832 (Portugal, 2011): repetitions number 9; 5) PSA_2F (France, 2010): repetitions number 9; 6) PSA_18875 (New Zealand, 2011): repetitions number 9; 7) PSA_23664 (South Korea, 1989): repetitions number 3; 8) PSA_K2 (South Korea, 1997): repetitions number 3; 9) PSA_CH2010-6 (China, 2010): repetitions number 9; 10) PSA_K2T (Turkey, 2011): repetitions number 6; 11) PSA_JILO4 (China, 2012): repetitions number 5.

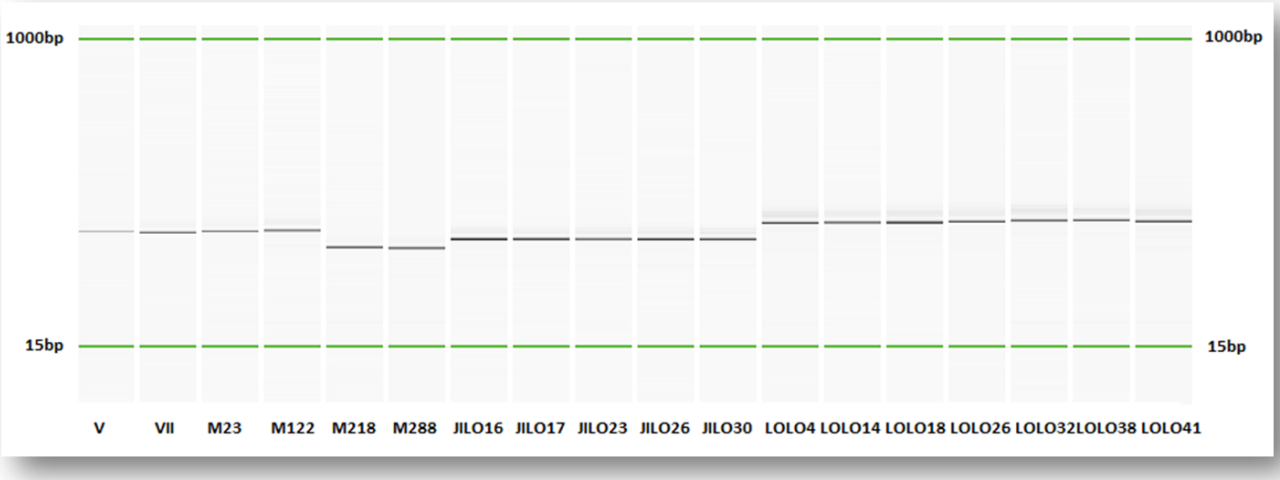
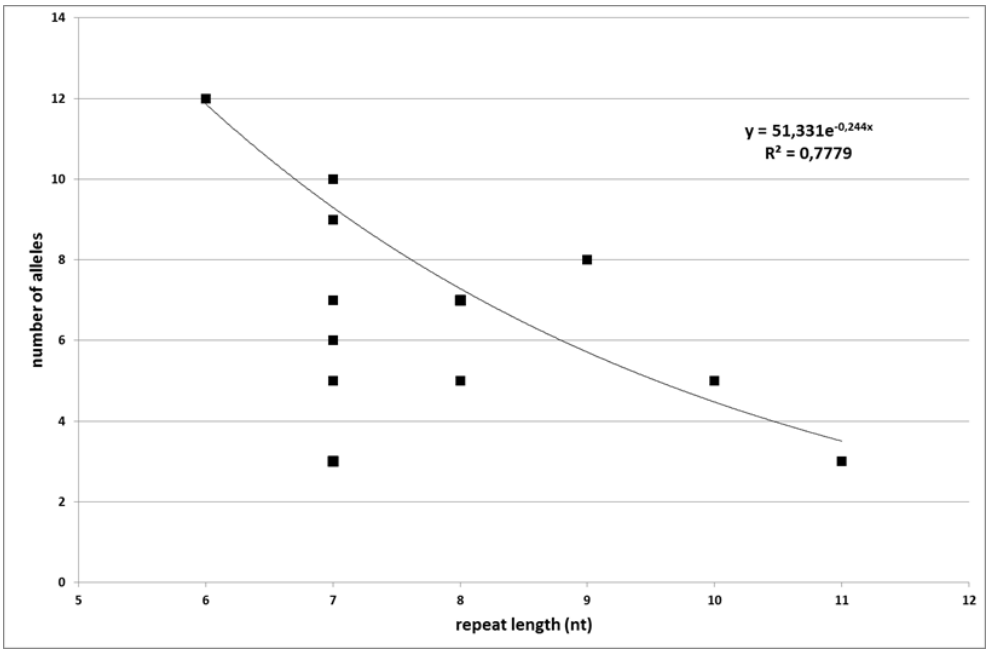


Fig. 7 Psa-10 on representative selection of Chinese Psa strains coming from several region run by OM700 method of QIAxcel with 15-1000 bp alignment marker. Lanes 1-18: 1-4) PSA_CH2010-5, PSA_CH2010-7, PSA_M23, PSA_M122 (Shaanxi, 2010): repetitions number 14; 5-6) PSA_M218, PSA_M288 (Shaanxi, 2010): repetitions number 9; 7-11) PSA_JILO16, PSA_JILO17, PSA_JILO23, PSA_JILO26, PSA_JILO30 (Anhui, 2012): repetitions number 12; 12-18) PSA_LOLO4, PSA_LOLO14, PSA_LOLO18, PSA_LOLO26, PSA_LOLO32, PSA_LOLO38, PSA_LOLO41 (Guizhou, 2012): repetitions number 16.

Fig. 8 Inverse correlation between TRs single unit length and numbers of observed alleles.



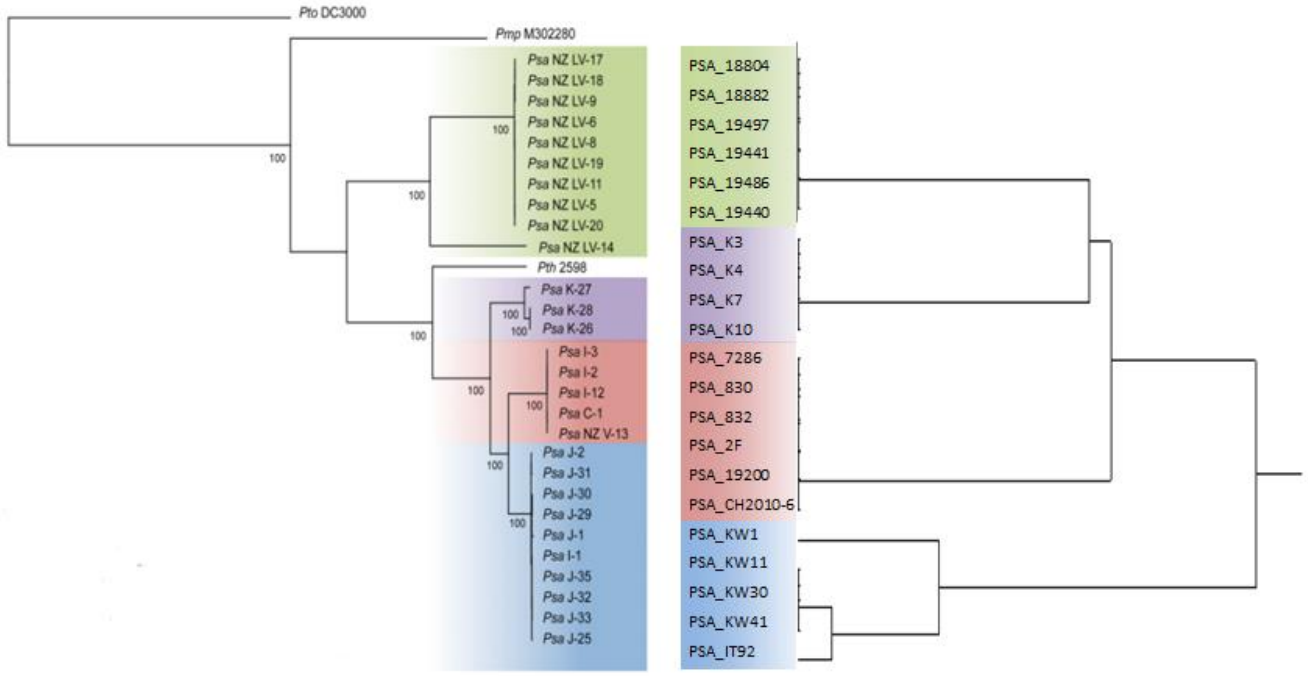


Fig. 9 UPGMA dendrogram derived from elaboration with the "diversity panel" of 6 loci on selected Psa (on the right) compared to that generated by the analysis of more than 15.000 SNPs (on the left) (McCann, 2013).

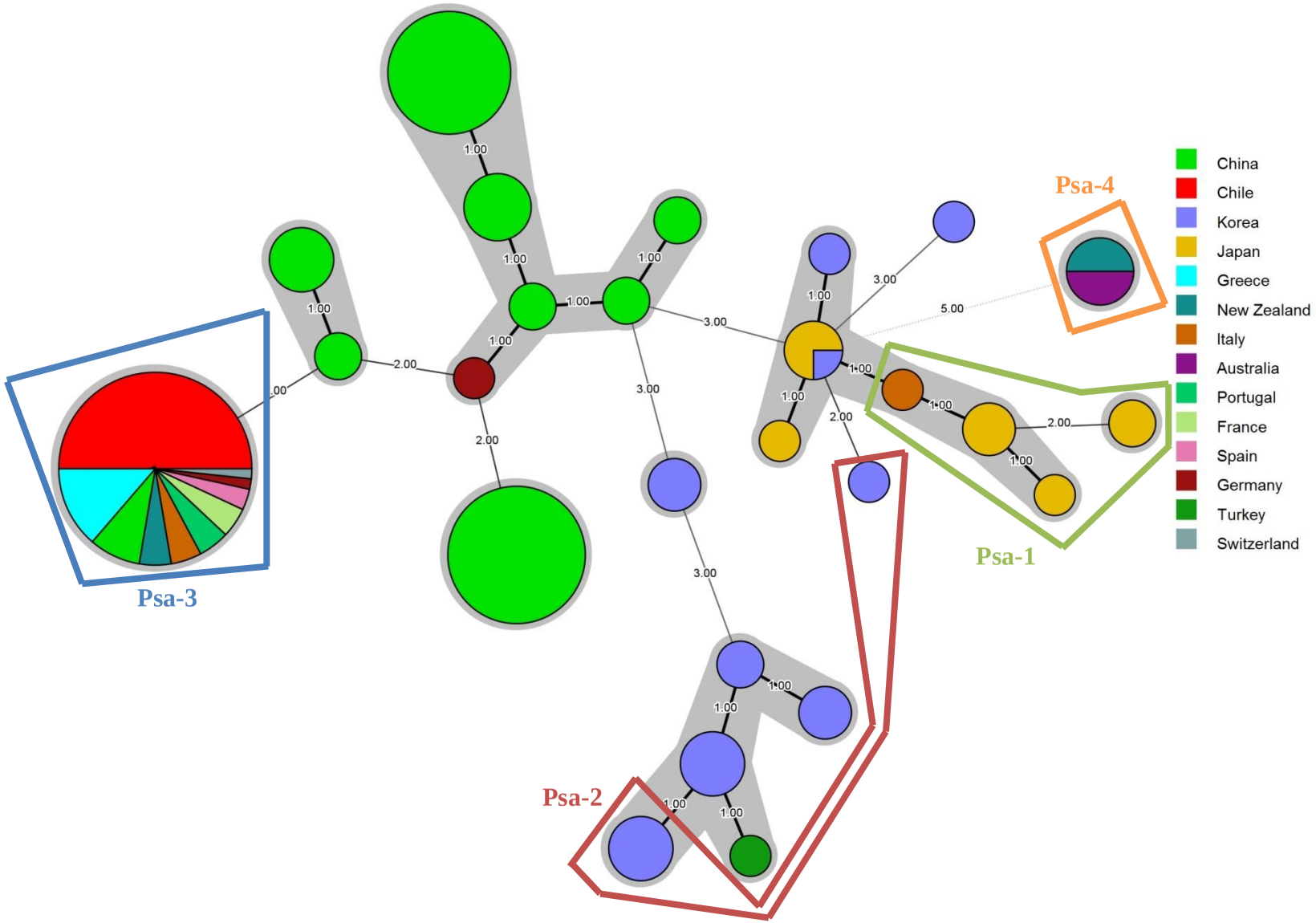


Fig. 10 Minimum Spanning Tree (MST) deriving from "diversity panel" analysis on the whole set of 168 Psa. Biovars as defined by Chapman (2012) are delimited by continuous lines encompassing one or more CCs (represented as grey shades). Each circle represents a group of strains with identical MLVA haplotype and its dimension is proportional to the number of strains included. The color of the circle corresponds to the geographical origin of the strains at country level.

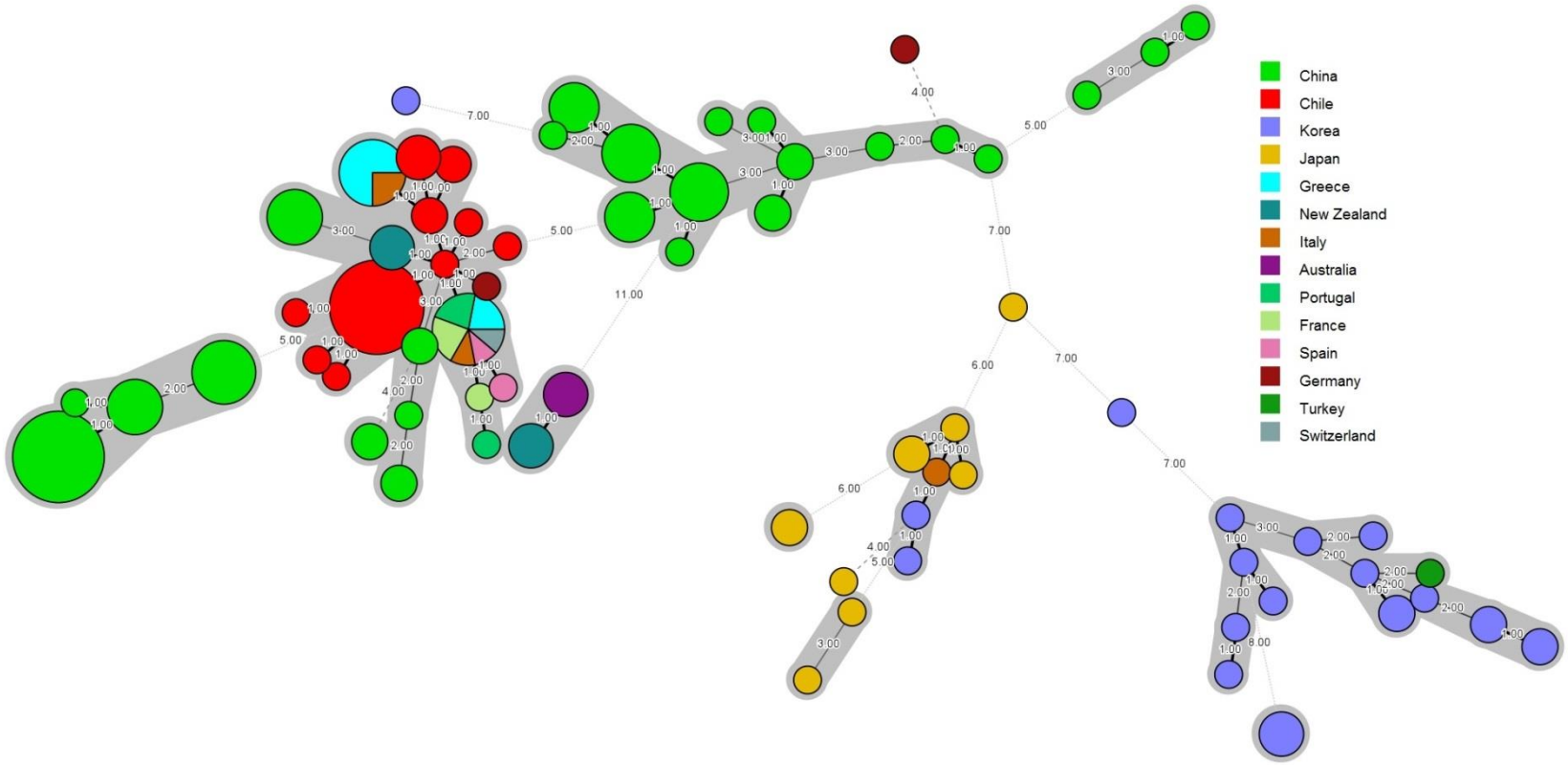


Fig. 11 MST deriving from data of the whole 13-loci MLVA panel on 168 Psa strains. Each circle represents a group of strains with identical MLVA haplotype and its dimension is proportional to the number of strains included. The color of the circle corresponds to the geographical origin of the strains at country level. The grey shaded areas represent the clonal complexes (CC), that were defined as those groups of strains having each other at maximum 3 VNTR loci of difference on 13 (about 23%). The numbers long the branches correspond to the number of differences between the linked group of strains.

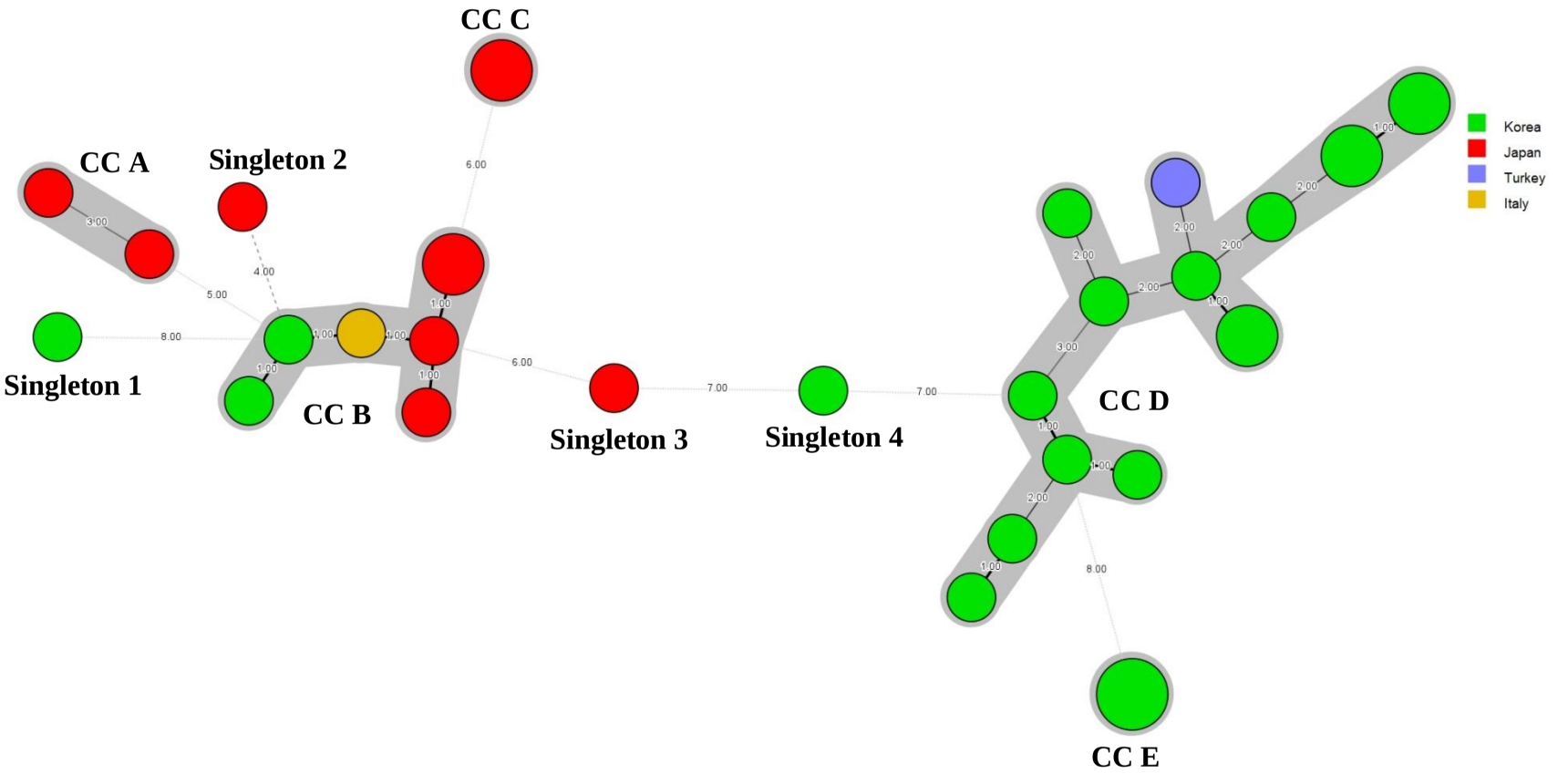


Fig. 12 MST from whole MLVA panel applied on Japan-Korea group. Each circle represents a group of strains with identical MLVA haplotype and its dimension is proportional to the number of strains included. The color of the circle corresponds to the geographical origin of the strains at country level. The grey shaded areas represent the clonal complexes (CC), that were defined as those groups of strains having each other at maximum 3 VNTR loci of difference on 13 (about 23%). The numbers long the branches correspond to the number of differences between the linked group of strains.

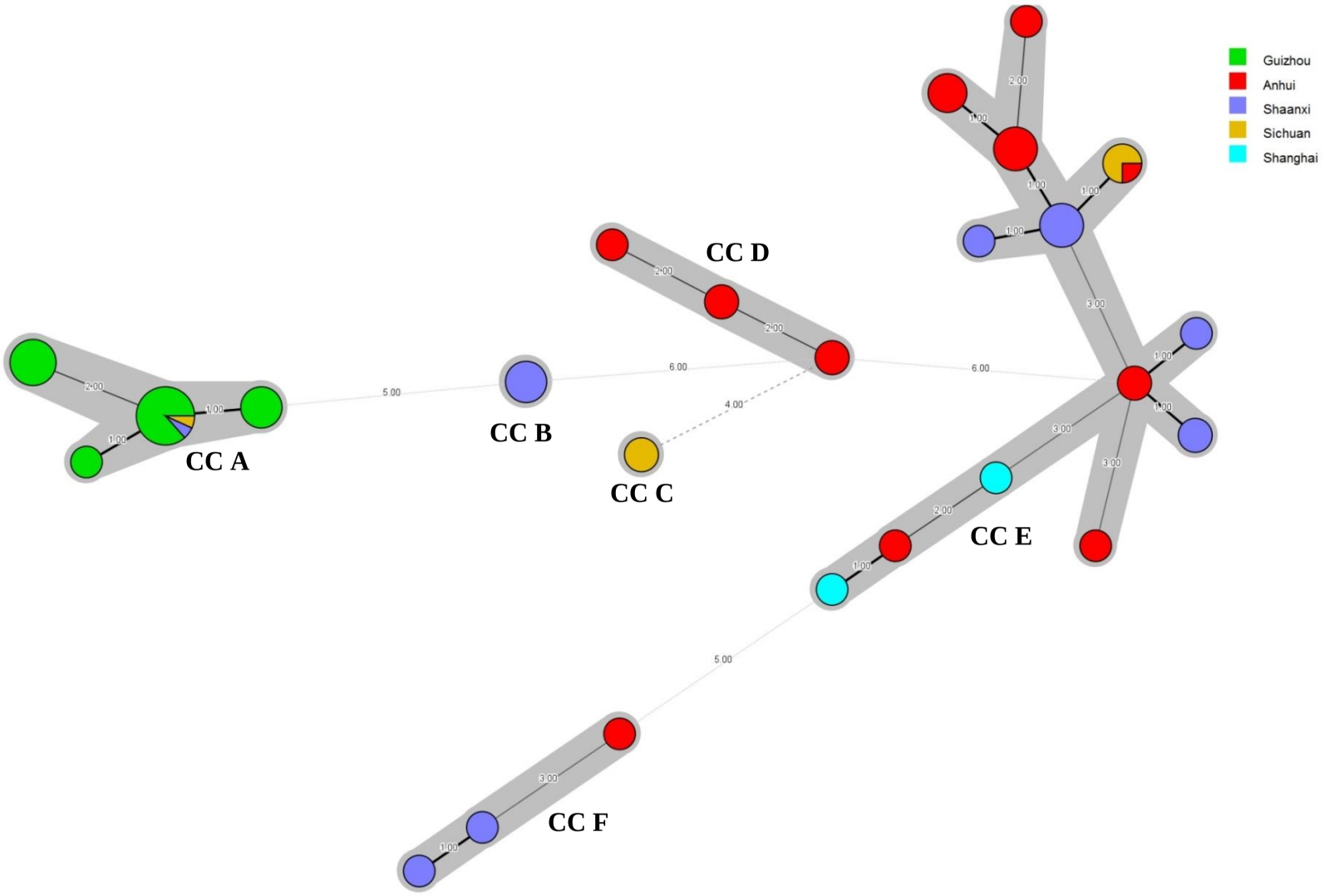


Fig. 13 MST from whole MLVA panel applied on China group. Each circle represents a group of strains with identical MLVA haplotype and its dimension is proportional to the number of strains included. The color of the circle corresponds to the geographical origin of the strains at province level. The grey shaded areas represent the clonal complexes (CC), that were defined as those groups of strains having each other at maximum 3 VNTR loci of difference on 13 (about 23%). The numbers long the branches correspond to the number of differences between the linked group of strains.

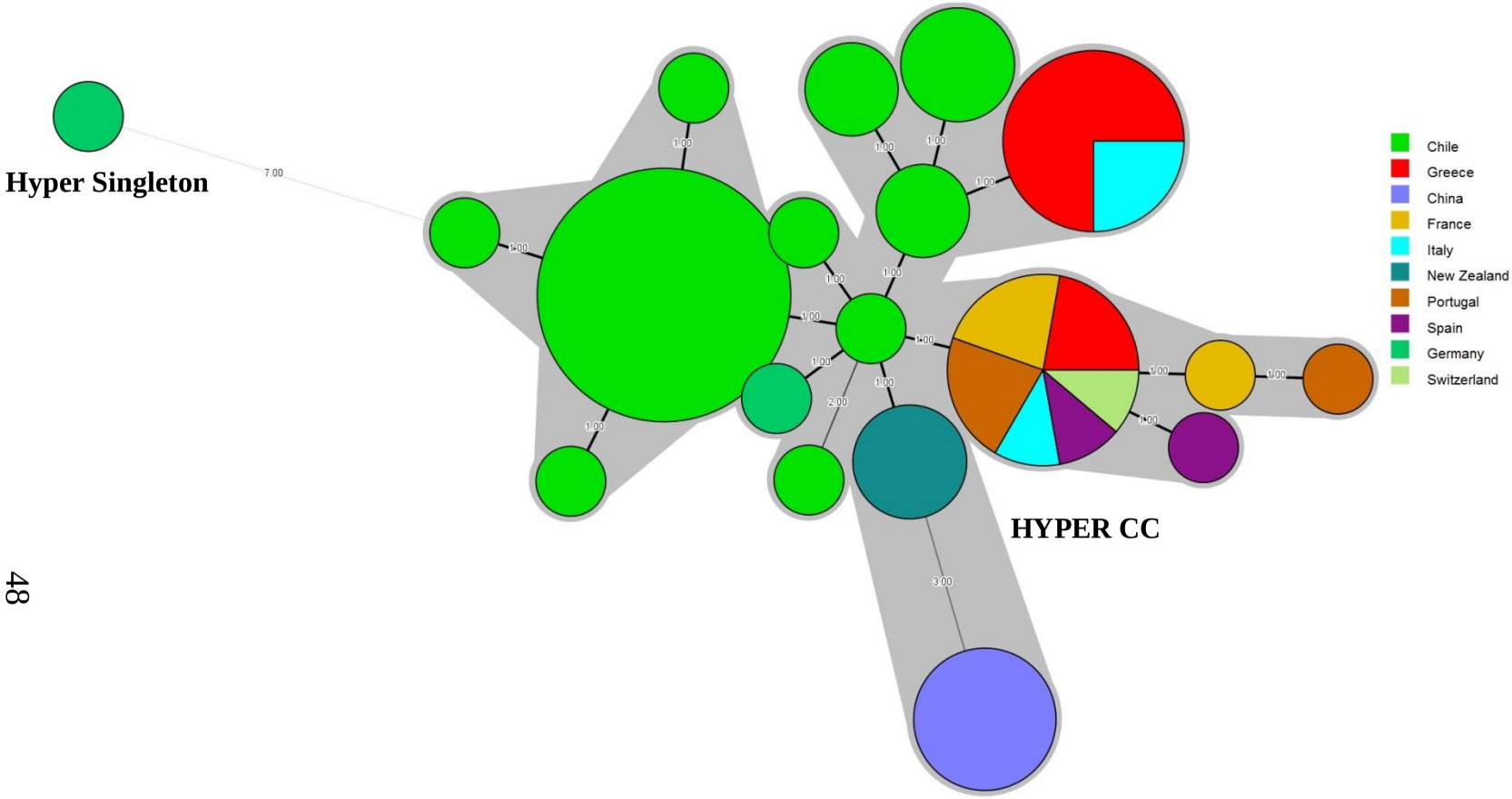


Fig. 14 MST from whole MLVA panel applied on Hypervirulent group. Each circle represents a group of strains with identical MLVA haplotype and its dimension is proportional to the number of strains included. The color of the circle corresponds to the geographical origin of the strains at country level. The grey shaded areas represent the clonal complexes (CC), that were defined as those groups of strains having each other at maximum 3 VNTR loci of difference on 13 (about 23%). The numbers long the branches correspond to the number of differences between the linked group of strains.

Fig. 15 UPGMA on Japan-Korea group. Clonal complexes as defined by MST with 13 loci were reported to highlight the relationships with similarity clusters, indicated by numbers.

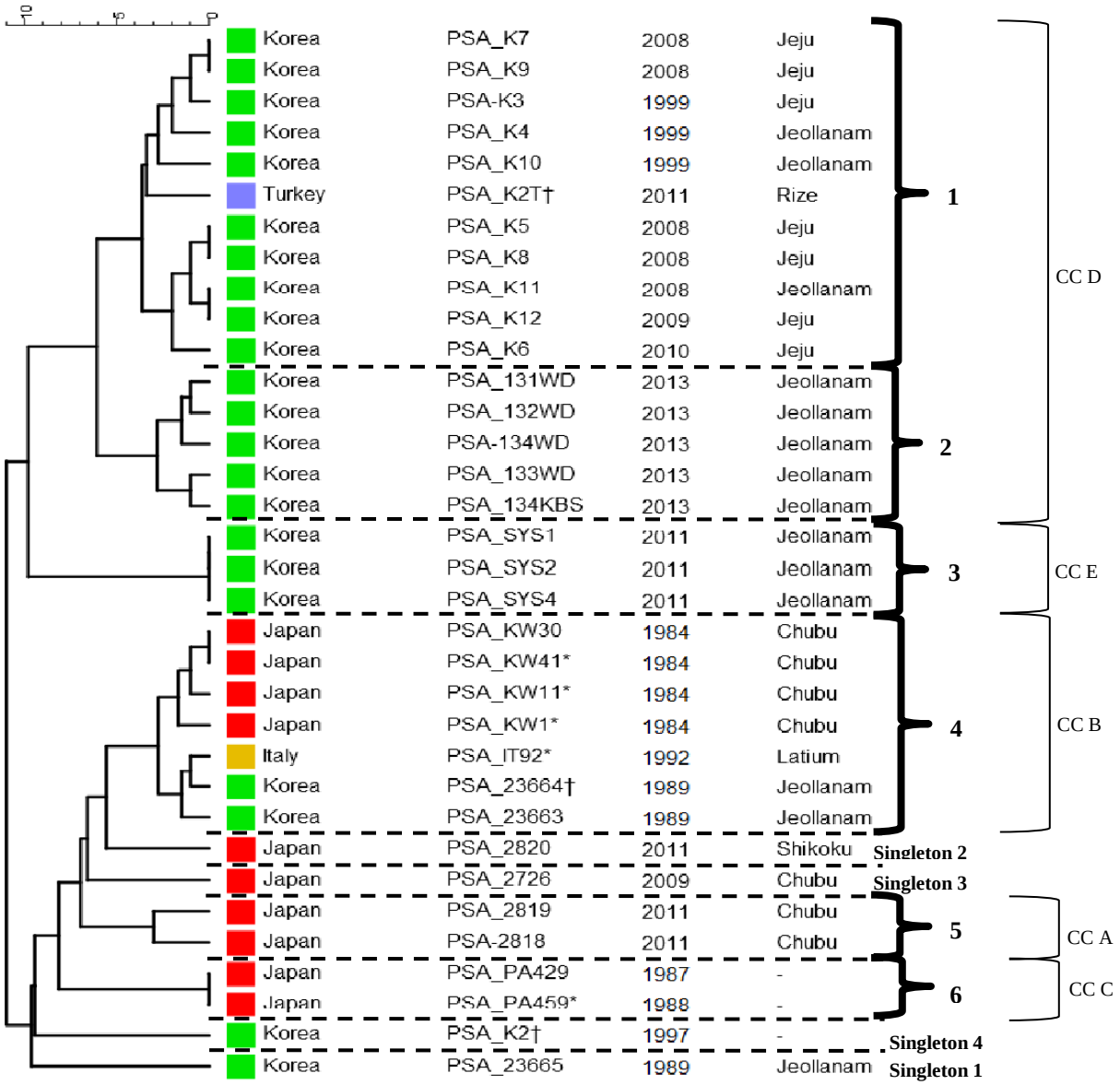


Fig. 16 UPGMA on China group. Clonal complexes as defined by MST with 13 loci were reported to highlight the relationships with similarity clusters, indicated by numbers.

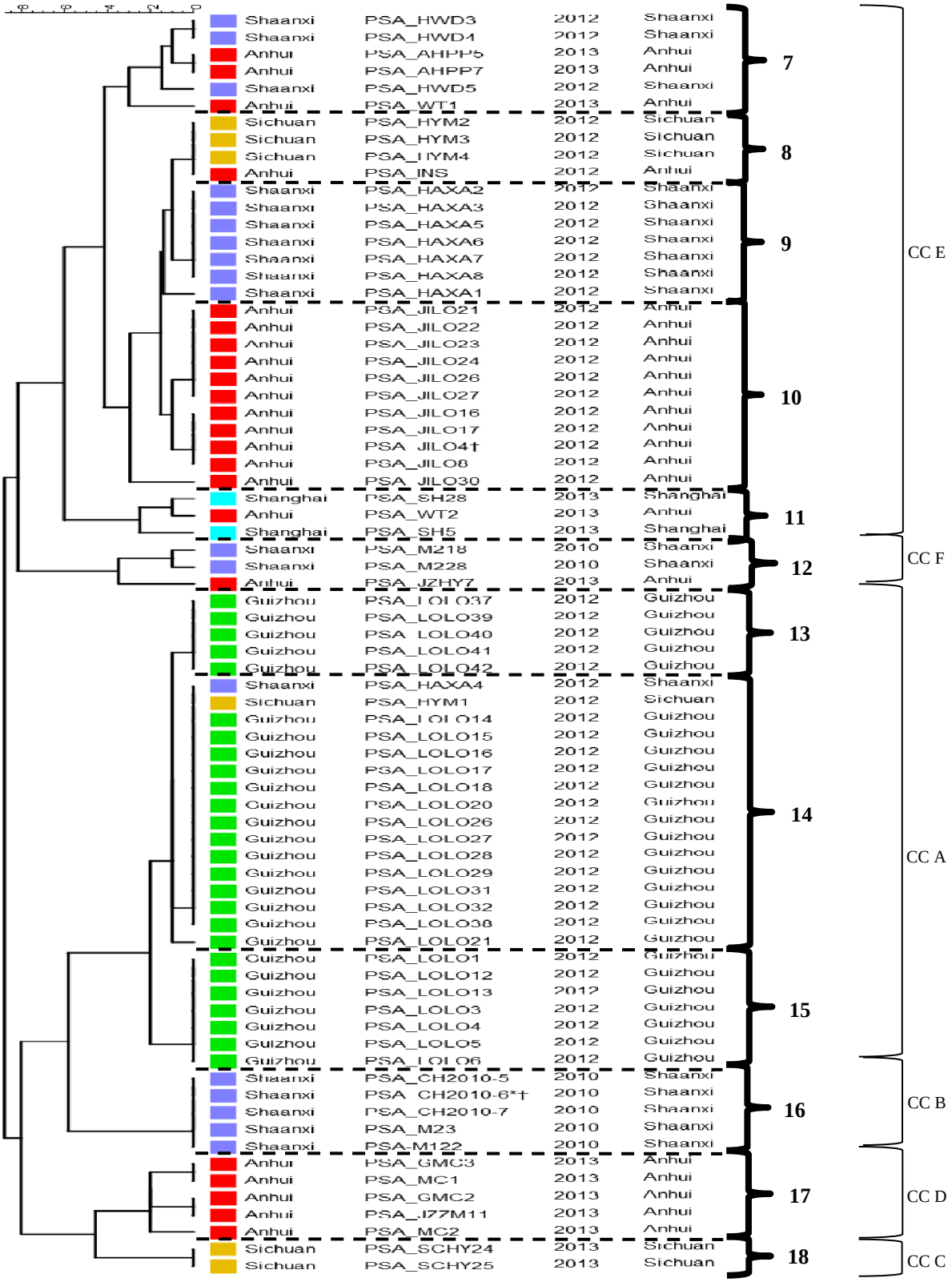
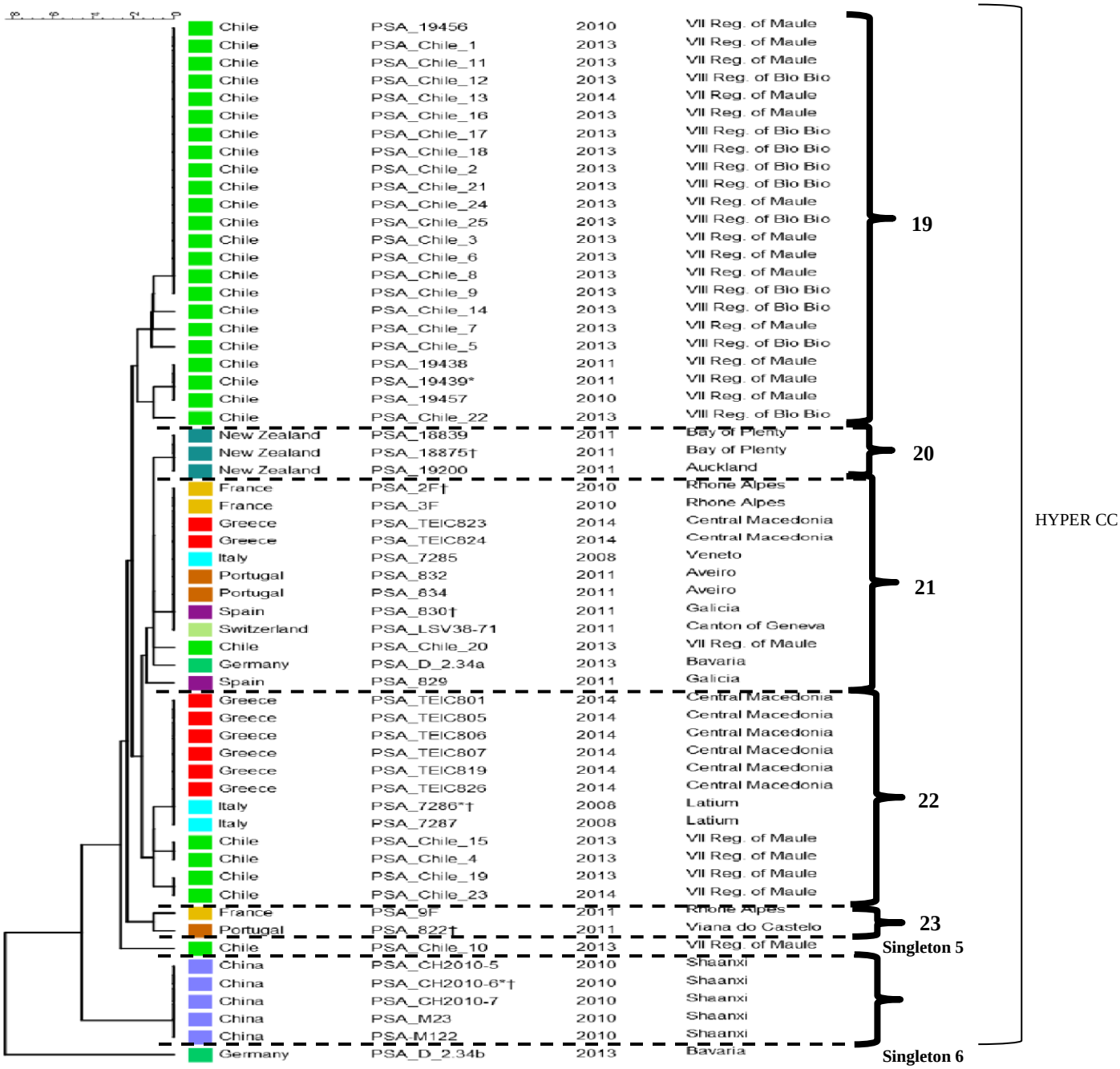


Fig. 17 UPGMA on Hypervirulent group. Clonal complexes as defined by MST with 13 loci were reported to highlight the relationships with similarity clusters.



6. DISCUSSION & CONCLUSIONS

The bacterial canker of kiwifruit caused by the *Pseudomonas syringae* pv. *actinidiae* was described in detail for the first time in Japan at the end of 80's, but this disease was very likely existing in the Asiatic area since long time before. In fact, the genus *Actinidia*, counting more than 60 species, is native to Central-Southern China, where it has been declared National Fruit, and China presumably it is also the natural area of coevolution of its pathogens, including Psa. However, even if damages from this pathogen were reported, these have never been described as destructive and Psa has been controlled and managed successfully in Asiatic orchards. But then, the introduction of kiwifruit on international markets had a great success in the last decades, and there was an astonishing spread of kiwifruit cultivation in New Zealand, Europe (with Italy in the lead) and South America, mainly in Chile. It is not a case that these 3 countries are to date the biggest producer of kiwifruit in the world, apart China.

The kiwifruit, as a general rule, was known as a robust and vigorous plant, with rare cases of severe damages due to microbial pathogens; but, suddenly, in 2008 many plants emerged in the main area of cultivation of kiwifruit in Italy (Latina Province), that showed exceptionally heavy symptoms. Immediate investigations identified Psa as causal agent of the disease (Balestra, 2009); since the first studies it was clear that this new epidemic was related to a new, very aggressive population of Psa, never reported before and drastically different from the previously known ones. Many researches promptly have followed and many approaches were attempted by the scientific community in order to clarify how the Psa is structured in terms of sub-populations and aiming to understand the origin of these. All the typing results, by RAPDs, rep-PCR, MLST of housekeeping genes, effector genes, etc. have leaded to the conclusion that at least 4 different types of Psa exist: an original population of Japanese strains, that includes also the Italian strains isolated in 1992; a Korean population formed by strains isolated at the end of 90's, the present population of very aggressive strains isolated after 2008 in Europe, New Zealand and Chile; and the low virulent strains of Psa, retrieved only from New Zealand and Australia.

Only the most recent papers approaching genome analysis were able to evidence some minor but crucial differences within the virulent population of Psa that is thus far struggling the kiwifruit orchards all over the world.

But in all of these studies the samples under investigation were seriously limited in terms of number and assortment of strains for what concerns geographical origin and period of isolation.

In this work both these thoughtful limits are at least overtaken. As a matter of fact, this is the first analysis that considers a more than conspicuous collection of Asiatic strains (74 Chinese strains from 5 different areas of cultivation, isolated from 2010 to 2013; 10 Japanese strains from 2 areas, isolated from 1984 to 2011; 22 Korean strains from 2 areas isolated from 1989 to 2013), together with an exhaustive selection of 54 strains from all the part of the world where the last virulent form of Psa was reported (22 from 7 European Countries isolated from 2008 to 2014; 3 from 2 areas in New Zealand isolated in 2011, 29 from 2 areas in Chile isolated from 2010 to 2014 and 1 from Turkey isolated in 2011), 6 strains of the low virulent type (3 from New Zealand and 3 from Australia) and a single strain from the sporadic report in Italy in 1992. Unfortunately, it was impossible to include also strains belonging to the newly determined biovar-5 in Japan (Sawada, 2014).

Understandably, a whole genome analysis of such a number of strains is unconceivable and an alternative method is definitely needed; this technique needs to be simple, cheaper, fast, robust and easily reproducible even at inter-laboratory level, but it should have contemporaneously a very high resolution power that make it able to weigh small but significant differences.

The MultiLocus VNTR Analysis (MLVA) has all these features and this is the reason why in the last years it has become a standard of particular efficacy in evaluating the population structure and dynamics of bacterial pathogens, both in animal and human diseases (Pourcel, 2004; Vergnaud, 2009; Pourcel, 2011b; Sobral, 2012).

The availability of WGS sequences from Psa strains of different origins permitted to develop a MLVA assay. The preliminary analysis involving just 6 on 13 VNTR loci, overall named “diversity panel”, succeeds in accurately reproducing Psa population structure already described in several previous works using MLSA (Chapman, 2012) or genome-wide SNPs analysis (McCann, 2013), but with additional advantages in terms of cost, time and execution difficulties, since MLVA requires just the common equipment to carry out standard PCR experiments. Moreover, by considering only 6 VNTRs, resolution is certainly higher than that of the above mentioned methodologies, as 9 different clonal complexes and 2 singletons are detected during this preliminary phase.

The 13 VNTR loci used in the whole MLVA scheme have generated 72 different haplotypes among 168 Psa strains; and, noticeably, the structural scheme of the whole Psa obtained from this analysis, reveals that at least 11 different clonal complexes plus 5 singletons are present and points out the existence of a much more complex arrangement for this pathogen than thus far reported.

The first observation is that China is the Country where the widest genetic variability resides; this was evidently expected due to the millenary coevolution of host and pathogen, but it was never scientifically demonstrated before. Even the most recent paper about China population of Shaanxi Province (Zhao, 2013), analyzing strains representative for more than 300 strains collected on various kiwifruit species and cultivars in 2010-2011 by 16S and rep-PCR fingerprinting, didn't find differences between Chinese strains and Italian/New Zealander ones. On the opposite, our results evidenced that strains from China constitute, or are part of, 6 different clonal complexes, "China CC A", "China CC B" (actually included in the "Hyper CC"), "China CC C", "China CC D", "China CC E" and "China CC F" with interesting remarks on each of them.

The clonal complex "China CC E" deserves a particular attention; as a matter of fact, it seems to be the one with a widest geographical distribution in China due to its contemporaneous presence in 3 areas (Anqing, Xi'an, Deyang and Jiading) that are thousands of kilometers far each other, on different species of *Actinidia* (*A. chinensis* and *A. deliciosa*), as well as on different cultivars (JinFeng, Hayward and Hong Yang) and on endemic plants (*Pauwlonia fortunei (Seem.) Hemsl*, *Philagra quadrimaculata Schmidt*). The Psa strain isolated in Anqing area from the possible vector insect, identified as *Cicadellidae*, belongs to this clonal complex too.

The second clonal complex "China CC A" is conversely constituted almost only by strains from Guiyang area, in Anhui province, at least distant about 700 km from the other closest Chinese area (Deyang); this clonal complex unexpectedly includes two exceptions: one strain from Xi'an (PSA_HAXA4) and one from Deyang (PSA_HYM1), that share exactly the same VNTRs haplotype with 13 strains from Guiyang area. Their presence suggests that this haplotype is sporadically present in few other areas.

The third clonal complex, "China CC D", includes 5 isolates all hailing from Jinzhai area in Anhui province, but from various hosts: besides Actinidia deliciosa, in this group Psa resident on the two wild interhost species *Setaira viridis (L.) Beauv.* and *Alternanthera philoxeroides (Mart.) Griseb.* are found. Separated by only 4 differences and exclusively linked to the last clonal complex is

"China CC C", constituted by the only 2 strains from Pengzhou in Sichuan province, distant about 50 km from the nearest area of kiwifruit cultivation (Deyang in Sichuan province).

"China CC F" is formed by 3 strains, 2 from XianYang and Baoji in Shaanxi province, distant each other about 50 kilometers, and 1 from Jinzhai in Anhui province. The remaining Chinese 5 strains, again from Shaanxi Province (from Sheng and Baoji areas, just 10 km far apart), are included in the "China CC B", together with all and only the virulent strains of the last epidemic (European after 2008, Chilean and virulent New Zealander). Therefore, this clonal complex is of key relevance to understand the origin of the current highly destructive and worldwide distributed epidemic; as a matter of fact, all the strains, apart Shaanxi Chinese strains, belong to what is currently described as Psa-3 haplotype (Chapman, 2012). These data strongly support all the previous suggestion about a possible Chinese origin of the above cited epidemic (Mazzaglia, 2012; Butler, 2013).

Observing Chinese results from another point of view, can be perceived that the largest genetic variability in a single area is by far ongoing in the Shaanxi province. In a radius of about 50 kilometers we retrieved strains belonging to as much as 4 different clonal complexes ("China CC A", "China CC B", "China CC E" and "China CC F"). This datum can be explained considering that in this province, and mainly in Mei county, there is the China's largest cultivated area of kiwifruit, that in 2011 has reached totally about 600 km^2, with a production of 700,000 tons, being worth of a third of the global Chinese kiwifruit production. Moreover, Shaanxi kiwi fruits, apart covering most of the internal market in China, are also exported to Russia, Canada, U.S., Japan, ROK, EU, Taiwan and countries in Southeast Asia. (data from the article "Kiwi Fruit Yields Top in Shaanxi Fruit" published on the website of the Department of Commerce, Shaanxi Province, http://new.shaanxiinvest.gov.cn/tzdt/show.asp?id=3991). Furthermore, here are located most of the companies producing and commercializing kiwifruit, pollen and vegetative materials not only for Chinese market but for international export too. The hypothesis of a transfer of contaminated material from China to other Countries has to be carefully pondered: visually healthy fruits that are commercialized, are currently considered as free of bacterial contamination risk (Stefani, 2011); a moderate peril is instead linked to contamination of pollen (Biondi, 2013; Gallelli, 2011; Vanneste, 2011b), that is subjected to a noteworthy national and international marketing, due to its role in ameliorating fruit size and shape. Actually, the risk of the pathogen's spread via contaminated vegetative material is much more concrete; the well-known PSA biotic behaviour of being able to colonize the vascular system of kiwifruit plants without producing any symptom, at least for a more

or less definite period of time (Vanneste, 2011c), makes the commercialized plantlets the critical risk point for the diffusion of the pathogen on a large scale, both national and international.

Another critical point is represented by the genetic diversity encountered in Psa strains isolated on non-host endemic plants: VNTRs constituting the proposed MLVA panel seem not to be affected by plant species where the bacterium resides, but rather they follow a geographic and chronological model; nevertheless, wild plants could be seen as a reservoir of genetic variability and a sort of "temporary host" for Psa that should deserve more attention and monitoring activity.

For what concerns the rest of Asiatic strains, from Japan and Korea, MLVA results suggest that their grouping can be considered as outcome of a mix of geographic origins and isolation dates.

The clonal complexes "Japan-Korea CC B" and "Japan CC C", each other somehow linked (6 additional VNTR loci of difference) are essentially constituted by Japanese strains isolated from 1984 to 1988, with the addition of 2 Korean strains isolated in 1989 and the Italian strain isolated in 1992 in "Japan-Korea CC B". This seems to be the bunch of the earliest recognized Psa strains, all coming from Asiatic area, and mainly from Japan. Also, the Japanese strains of "Japan CC A" , isolated in 2011, and some singletons again from Korea (1989 and 1997) and Japan (2009 and 2011), are observed in relative connection to the previous CCs, as demonstrated by their overall fitting into the MLVA similarity cluster 1. No other Japanese strain can be detected elsewhere in the MST clustering. In this quite loose group all the Japanese strains referable to the depiction of the pathovar by Takikawa and colleagues (1989), the oldest Korean strains from 1989 to 1997 and the most recent Japanese strains ("Japan CC A" and singletons) are each other related. The resulting conclusion is that Japanese population has evolved by its own ancestors, together with the older Korean strains.

On the other side, the large majority of Korean strains (18 on 22), all isolated between 1999 and 2013 from a lot of different areas of that country, constitute another two groups, "Korea CC D" encompassing the greater number of isolates (15 on 22) and "Korea CC E", constituted only by 3 strains isolated in Goheung-gun in the region of Jeollanam all in 2011 (similarity clusters 1 and 2). Interestingly, these Korean strains are not in connection with those isolated previously (1984-1987). In consequence, both MST and UPGMA clearly advise that the most recent Korean strains have arisen from a different genetic lineage, of greater aggressiveness and pathogenicity, that is now well established in Korea where is still causing serious and alarming damages, particularly in the Jeju Province (Koh, 2010). This is a news on which to pay serious attention, in order to reduce to the

minimum the risk of diffusion of another aggressive pathotype, currently limited within Korean borders. In such sense, it has to be highlighted that the unique Turkish strain available from the report of Bastas and Karakaya (2012) belongs to "Korea CC D" too, and it calls into question if this is the first case of its international spreading.

Finally, the "Low Virulent CC" is formed only by strains both from New Zealand and Australia and is by far the most divergent clonal complex, sharing very few VNTR loci (2 on 13) with other CCs. Our MLVA data strongly support the assumption that the taxonomical position of this group have to be deeply revised, taking it well apart from the real Psa, as already suggested by Butler and colleagues, that proposed to renamed these strains as PsD, for *Pseudomonas syringae deliciosa* (Butler, 2013), or by Cunty group, hypothesizing the name *Pseudomonas syringae* pv. *actinidifoliorum* pv. nov (Cunty, 2014).

In this study, the MLVA applied on the widest worldwide Psa collection to date simultaneously analyzed resulted to be very effective in distinguishing Psa subpopulations and unraveling their complicate relationships in terms of geographical origins, but also in conjunction to their isolation dates and even their aggressiveness. It was demonstrated how much larger is the genetic assortment of Psa worldwide in respect to what was up to now reported, but contemporaneously confirmed that the most virulent form of Psa has reference to a very close genetically clump of Chinese strains, only from the Shaanxi Province. And it is almost evident that the devastating clonal population had succeeded as a consequence of its introduction in new environment and on new kiwifruit plants devoid of naturally evolved defenses.

In such sense, even if the pathogenicity of the other Asiatic strains, both Chinese, Korean and Japanese, was not still comparatively evaluated, it is however plausible that this huge amount of genetic variability can even so be considered as a reservoir of (pathogenicity) genes that could make them quickly adaptable and perilous if new favourable conditions would turn up suddenly (advent of new cultivars, introduction in new environments, etc.).

Concerning the method, the resolution power of MLVA analysis resulted almost comparable to that of genomic studies, and the results fit almost perfectly with those obtained with genome sequencing, even if the target of MLVA is represented by sequences that are hardly analyzable with WGS sequencing. MLVA have several other advantageous key traits: it is cost-effective, ease to be reproduced in any laboratory where basic instruments for molecular analysis are present and doesn't require particular expertise to be carried out. Moreover, as ascertained in this study, VNTRs

included in the panel result to be very stable under laboratory conditions, so as to make them potentially exploitable in a worldwide inter-laboratory Psa surveillance system (James, 2014).

Last but not least, the VNTR panel presented here is not a close and stiff system; on the contrary it should be a starting point to be further improved in the future, by simply adding new VNTR loci, also running several alghoritms for TRs search (O'Dushlaine, 2006; Pellegrini, 2010), effective in resolving the complex arrangement of a worldwide diffuse pathogen like Psa.

Another VNTR approach that is still in evaluation, is represented by a sort of "progressive MLVA": it takes out from the hypothesis of using VNTR loci not altogether simultaneously, but starting instead with those at lower level of resolution and then, on the basis of results, choosing step by step the appropriate VNTR locus that permits to reach the final collocation in the whole Psa scenery, with a reduced number of amplifications.

In fact, our data statistically support the hypothesis that there is an inverse proportionality between the length of TRs and their variability, or in other words, their chance to be discriminative at different taxonomic level; that's why, also taking in proper account the astonishing growth of genome sequences publicly available and their increasing quality and accuracy, the probabilities to find out other VNTR loci with a discriminative efficacy variable according to the taxonomical level to be investigated, are rapidly growing up. In such sense, and considering how VNTR loci derived from the analysis of other *Pseudomonas syringae* pathovars (Gironde, 2012) were effective even for Psa, is therefore plausible that a wider approach to this fascinating technique could provide new important insights even to describe relationships among the whole *Pseudomonas syringae* complex.

Lastly, potential functional role of found VNTRs should be closely investigated; in fact over the role of markers for linkage mapping and forensic purposes initially covered thanks to their high polymorphism (Nakamura, 1987; Odelberg, 1989), they were later thoroughly studied also for the multiple functional roles carried out along the DNA. The human genome is a clear example of how much multitasking these sequences are: if they are inside to an intragenic region, it is highly probable that they are codifying essential and well-defined proteic motifs (Faux, 2005) and they represent an almost limitless possibilities of ameliorating genetic repertoire (Verstrepen, 2005). Very often VNTRs are associated to non-coding regions, where they accomplish a regulatory role: serotonine and dopamine transporters genes are well-known to be regulated by a 40 bp-VNTR, with the most common alleles showing 9-10 repetitions, functioning as transcriptional enhancer: it has been demonstrated that repetition number strongly conditions the susceptibility to some anxiety-

related disorders as schizophrenia, Parkinson's disease, drug and alcohol abuse (Michelhaugh, 2001).

Concerning bacteria, some works are available on VNTRs functionality with almost the entirety concentrated on human pathogenic microorganisms. *Mycobacterium tuberculosis* has been studied by epidemiology point of view for a long time applying MLVA panels; most recently, two VNTRs, VNTR0960c and VNTR4052, were tested for their capacity to regulate transcriptional activity on GFP, evidencing that VNTR0960c could promote the expression of the reporter gene, while VNTR4052 alone could not. However, VNTR4052 was needed for complementing the promoter activity of the downstream sequence (Tantivitayakul, 2010). Analogue works were carried out on *Streptococcus* (Whatmore, 2001), *Staphylococcus* (Hussain, 2008) and *Neisseria* (Van der Ende, 1995).

Since VNTRs may play an important functional task into bacterial cell and in general a key role into adaptability and evolution (Gemayel, 2010; Zhou, 2014), it could be useful and interesting verify potential functionality of repeated sequences of Psa genome, found in this work, to clarify any possible correlations with virulence degree or nutrition sources utilization.

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