Taxonomic relationships among species of *Pectobacterium* and description of two novel taxa within the heterogeneous species *P. carotovorum*, namely *P. carotovorum* subsp. *brasiliense* subsp. nov. and *P. aroideae* sp. nov.

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Abstract

The study provided the data to describe two new taxa in the genus *Pectobacterium,* namely *P. carotovorum* subsp. *brasiliense* and *P. aroideae*, and thereby made significant progress in the understanding of the heterogeneity within the genus and the *P. carotovorum* species. An in-depth characterization and analysis of forty-six bacterial strains from international culture collections and an additional thirty new strains from Syria, which were deposited in culture collections, was performed. All genomic data, including gene sequences were submitted to the NCBI GenBank to ensure their availability to the scientific community.

Further 41 strains were investigated using available genetic sequence data retrieved from the NCBI GenBank. The strain collections represented the *Pectobacterium* genus with its four previously reported species of *P. carotovorum*, *P. atrosepticum*, *P. betavasculorum* and *P. wasabiae* and the fifth species verified in this study of *P. aroideae*. Additionally, the species *P. carotovorum* was represented with its three subspecies of *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum* and the third subspecies verified in this study of *P. carotovorum* subsp. *brasiliense*.

The study utilized both standard and novel techniques which are accepted in the current systematic opinion. Amplified fragment length polymorphism (AFLP) and multi-locus sequence analysis (MLSA) of eight protein coding genes were performed to resolve the phylogenetic relationships within *Pectobacterium* spp. The AFLP analysis generated 925 clearly scorable DNA-fragments, and resolved five clusters within the *P. carotovorum* (PcI, PcII, PcIII, PcIV, and PcV).

The study demonstrated the advantage of MLSA as a taxonomic tool which can resolve phylogenetic relationships among a large set of strains and proved its suitability for species delineation. Around 3500 bp represented by each strain resolved the heterogeneity within the species *P. carotovorum* by differentiating the subspecies and delineating two *P. carotovorum* subsp. *brasiliense* clades as well as two *P. carotovorum* subsp. *carotovorum* clades. The Syrian strains did not display a monomorphic clade and were distributed over five phylogenetic clades in both MLSA and AFLP trees. The MLSA trees based on

individual genes suggest that horizontal gene transfer events occurred among strains belonging to different species within the genus.

The phylogenetic information was supported by the classification techniques of DNA-DNA hybridization and 16S rDNA. A complete sequence of 64 homologs of the 16S rDNA gene demonstrated polymorphism among the seven copies existing in the chromosome of a single strain. None of 16S rDNA homologs obtained from strains belonging to different subspecies was identical to one another, but two sequence types obtained from the same strain even placed distantly from one another in the phylogenetic tree. The study reported very low discriminatory values confirming that use of the 97 % threshold for differentiating species can not guarantee species identity. The speciation events suggested by the AFLP and MLSA trees topologies were supported by DNA-DNA hybridization. The DNA-DNA hybridization confirmed that *P. carotovorum* subsp. *brasiliense* (cluster Pc IV) is a different species from *P. wasabiae*, *P. betavasculorum and P. atrosepticum,* with 28 %, 35 %, and 55 % similarity values, respectively, but is a member of the *P. carotovorum* species with 73-77 % similarity values. Additionally, DNA-DNA hybridization values ranged from 30.4 to 64.1 % between strains of *P. aroideae* (cluster PcV) and type strains of other *Pectobacterium* spp.

Carbohydrate utilization using GN Biolog microplate and conventional biochemical analyses were used to determine the phenotypic characteristics of the new taxa. All strains of the *P. carotovorum* subsp. *brasiliense* ferment lactose, tolerate 5 % NaCl, grow at 37 ^{o}C, and utilize D-trehalose and D-melibiose and can not utilize glucose-1-phosphate, malonic acid, dextrin and D-arabitol. The *P. aroideae* strains were also tested and it was demonstrated that strains of the *P. aroideae* differed from *P. carotovorum* in their utilization of dextrin, glucuronamide, and α-ketoglutaric acid. For both *P. carotovorum* subsp. *brasiliense* and *P. aroideae* type strains were designated as LMG21371 ^{T} and LMG 2417^{T,} respectively.

Key words: AFLP, MLSA, *Pectobacteria*, Syria, monomorphic, cluster, phylogenetic, NCBI GenBank, sequence, classification, DNA-DNA hybridization, threshold.

Zusammenfassung

Ziel dieser Arbeit war die Beschreibung zweier neuer Arten in der Gattung *Pectobacterium*, *P. carotovorum* subsp. *brasiliense* und *P. aroideae*. Dabei wird die Heterogenität innerhalb der Gattung *P. carotovorum* und zwischen den einzelnen Arten verdeutlicht. Die Arbeit enthält detaillierte Charakterisierungen und Analysen von 46 Bakterienstämmen aus internationalen Sammlungen und ergänzend 30 neue Stämme aus Syrien, die in Erhaltung genommen wurden. Alle genomischen Daten inklusive der Gensequenzen wurden bei der NCBI GenBank eingereicht, um sie der Wissenschaft zur Verfügung zu stellen.

Weitere 41 Stämme wurden mit Hilfe vorhandener Gensequenzen der NCBI GenBank untersucht. Die Sammlung der hier beschriebenen Stämme repräsentiert die Gattung *Pectobacterium* mit den bereits vier bekannten Arten *P. carotovorum*, *P. atrosepticum*, *P. betavasculorum* und *P. wasabiae* und wird jetzt durch die fünfte Art *P. aroidae* ergänzt. Zusätzlich wird die Art *P. carotovorum* mit den drei Unterarten *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum* und der dritten in dieser Arbeit bestätigten Unterart *P. carotovorum* subsp. *brasiliense* beschrieben.

In den Untersuchungen wurden sowohl Standardmethoden als auch neue Methoden verwendet, die allgemein anerkannt und reproduzierbar zur Unterscheidung bis unterhalb die Speziesebene sind. Amplified fragment length polymorphismn (AFLP) und Multiple Sequenzanalyse (MLSA) von acht protein-codierenden Genen wurden zur Bestätigung der phylogenetischen Beziehungen innerhalb der *Pectobacterium* spp. durchgeführt. Die AFLP-Analysen generierten 925 erkennbare DNA-Fragmente und fünf Cluster innerhalb *P. carotovorum* (PcI, PcII, PcIII, PcIV und PcV).

Diese Arbeit verdeutlicht den Vorteil der MLSA als Methode für taxonomische Untersuchungen zu phylogenetischen Beziehungen innerhalb einer großen Anzahl von Stämmen und zeigt die Verwendbarkeit für die Abgrenzung einzelner Arten. Jeder Stamm wird durch ca. 3500 bp beschrieben, die die Heterogenität innerhalb der *P. carotovorum* Arten zeigen und die Unterarten *P. carotovorum* subsp. *brasiliense* und *P. carotovorum* subsp. *carotovorum* in jeweils zwei Cluster unterteilen. Die syrischen Stämme zeigten keine monomorphologischen Cluster und waren in fünf phylogenetische Cluster sowohl in

den MLSA- als auch den AFLP-Stammbäumen aufgeteilt. Die MLSA basierten auf individuellen Genen, die horizontalen Gentransfer zwischen den Stämmen verschiedener Arten innerhalb einer Gattung vermuten lassen.

Die phylogenetischen Informationen wurden durch die klassifizierenden Methoden der DNA-DNA- Hybridisierung und der phylogenetischen Analyse des 16S rDNA-Gens abgesichert. Eine vollständige Sequenz von 64 Homologen der 16S rDNA Gene zeigte Polymorphismen innerhalb der sieben Kopien, die auf dem Chromosom eines Stammes liegen. Keiner der 16S rDNA Homologen, die zu unterschiedlichen Unterarten gehören, war identisch mit einem anderen. Genauso waren zwei Sequenz-Typen eines Stammes phylogenetisch nur entfernt verwandt. Die Ergebnisse zeigten sehr niedrige Unterscheidungswerte, die bei Gebrauch des 97 % Grenzwerts zur Unterscheidung der Arten, die eindeutige Differenzierung der Artenindentität nicht garantieren kann. Die Arteneinteilung durch die AFLP und MLSA konnte durch die DNA-DNA-Hybridisierung bestätigt werden. Diese verdeutlicht, dass sich *P. carotovorum* subsp. *brasiliense* (Cluster IV) eindeutig von *P. wasabie*, *P. betavasculorum* und *P. atrospeticum* bei Übereinstimmungswerten von 28 %, 35 % und 55 % abgrenzen lässt, aber mit Werten von 73-77 % Übereinstimmung zur Art *P. carotovorum* gehört. Ergänzend lagen die Werte der DNA-DNA-Hybridisierung zwischen Stämmen im *P. aroideae* (Cluster PcV) und anderen Stämmen der *Pectobacterium* spp. zwischen 30,4 und 64,1 % .

Zur Bestimmung der phänotypischen Merkmale der neuen Taxa wurden die Kohlenhydrat-Fermentierung mittels GN Mikroplatten und konventionellen biochemischen Analysen untersucht. Alle Stämme von *P. carotovorum* subsp. *brasiliense* fermentierten Laktose, tolerierten 5 % NaCl, wuchsen bei 37 °C, bauten D-trehalose und D-melibiose ab, konnten aber Glukose-1-Phosphat, Malonsäure, Dextrin und D-arabitol nicht spalten. Die *P. aroideae* Stämme wurden ebenfalls untersucht und zeigten Unterschiede im Vergleich zu den *P. carotovorum* beim Abbau von Dextrin, Glukuronamiden und α-Ketoglutarsäure. Die Arten *P. carotovorum* subsp. *brasiliense* und *P. aroideae* wurden als Stämme LMG21371^{T} beziehungsweise LMG2417^{T} bezeichnet.

Schlagworte: AFLP, MLSA, *Pectobacteria*, Syrien, monomorphe, Cluster, phylogenetische, NCBI GenBank, Sequenz, Klassifizierung, DNA-DNA-Hybridisierung, Grenzwerts.

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Abbreviations

λ	Lamda DNA
α	Alpha
A	Adenine
acnA	aconiate hydrase 1
AFLP	Amplified Fragments Length Polymorphisms
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CVP	Crystal Violet Pectate
CFBP	Collection Francaise des Bacteries Phytopathogenes
C	Cytosine
CFU	Colony Forming Unit
D.	Dickeya
DNA	Deoxyribonucleic acid
DDH	DNA-DNA hybridization
da	*dadantii*
dNTPs	Mix of equal concentration of the four deoxynucleotide triphosphates
DSMZ	German resource centre for biological material
E.	*Erwinia*
e.g.	example
et al	Et alii (and others)
GTR	General Time Reversible model
+G	Gamma distribution

Abbreviations

G	Guanine
G + C %	mol percentage of guanine and cytosine content
GN	Gram-negative
gapA	glyceraldehyde-3-phosphate dehydrogenase
GGD	genome-to-genome distances
HSPs	high-scoring segment pairs
h	hour
ICMP	International Collection of Micro-organisms from Plants
IBSBF	Biological Institute Culture Collection of Phytopathogenic Bacteria
icd	isocitrate dehydrogenase
ITS	Intergenic transcribed spacer
IGS	Intergenic Spacer
IJSEM	International Journal of Systematic and Evolutionary Microbiology
I	assuming that a certain fraction of sites are evolutionarily invariable
JKI	Julius Kühn Institute
i.e.	"that is; in other words"
LMG	Belgian Co-ordinated Collections of Micro-organisms
LB	Luria Broth medium
ml	Milliliter
Min	minute
mM	Millimolar
mltD	mannitol-1-phosphate 5-dehydrogenase
monocots	monocotyledones
mdh	malate dehydrogenase

MLSA	Multi Locus Sequence analysis
$MgCl_2$	Magnesium Chloride
McL	Maximum Composite Likelihood
ML	Maximum Likelihood
NCBI	National Center for Biotechnology Information
n.	number
nov.	novel
NCPPB	National Collection of Plant Pathogenic Bacteria
NaCl	Sodium chloride
ng	nanogram
NJ	Neighbour Joining
pl.	Plural
PCR	Polymerase Chain Reaction
proA	γ-glutamylphosphate reductase
pgi	glucose-6-phosphate isomerase
pmole	picomole
P.	Pectobacterium
RNA	ribonucleic acid
rpoS	RNA polymerase subunit sigma factor38
rpm	rotation per minute
RFLP	Restriction Fragments Length Polymorphisms
STM	Soya Tryptic Medium
subsp.	subspecies
sp.	species

Abbreviations

SCRI	Scottish Crop Research Institute
S	supplementary
SYM+G	symmetrical model plus gamma
T	Type strain
TIM+G	Transition model plus gamma
TN	Tamura and Nei
T	Thymin
TrNef+G	equal-frequency Tamura-Nei plus gamma
TIMef+)	equal-frequency transition model plus gamma
µg	microgram
UPGMA	majority-rule consensus trees
U	Unit
µl	microliter

1. General introduction

1.1. Bacterial species and speciation

Taxonomy is the art of identification, classification, and nomenclature of living organisms. Genetic exchanges and the intrinsic diversity make classification of organisms a major challenge in biology. One organism falls into a discrete cluster on the basis of phenotypic characteristics, can fall in another cluster due to its phylogenetic background (Cohan, 2001). Phenetic trees based on metabolic capabilities suggested that bacteria mediate the life between the Archaea and Eukaryotes (Aguilar *et al*., 2004). The bacterial kingdom is divided into pathogenic and non pathogenic prokaryotes, the pathogenic being separated into plant, human and/or animal pathogens, based on their pathogenicity. Moreover, different phylogeographical patterns which confer ecophysiological adaptations lead to increased diversity. However, their relatively small genomes of about 5 M bp makes bacteria the best model organisms for understanding speciation based on genetics. To date, more than sixteen hundred genomes (1661) have been sequenced (www.genomesonline.org). Comparative genomic analyses pave the way for understanding the pathogenicity and environmental adaptation to different hosts and geographic origins and consequently the speciation events.

Since 1970, DNA-DNA hybridization has been the classical method to define prokaryotic species. The phylogenetic definition of a species generally includes strains with DNA-DNA relatedness of approximately 70% or greater with 5°C or less $\Delta\ T_m$ (De Ley *et al*., 1970). Recently, average nucleotide identity (ANI) (Goris *et al*., 2007) was suggested as a replacement for DNA-DNA hybridization. Different molecular methods such as DNA sequencing, single nucleotide polymorphisms SNAPs (Achtman, 2008), DNA probe hybridization, and DNA typing methods including rep-PCR, RFLP and AFLP can be used to classify strains in appropriate groups and to resolve diversity. The G+C% mol content is considered in some studies as an important indication that discriminates the genome properties of a genomospecies.

Amplified fragment length polymorphism (AFLP) is a DNA fingerprinting method based on the digestion of total DNA with two restriction enzymes, followed by the ligation of restriction site-specific adapters and the amplification of a subset of fragments by PCR (Vos *et al*., 1995). This method is used for epidemiological studies and strain typing (Portier *et al*., 2006). The advantage of the AFLP methodology of targeting not only

conserved core genome but also accessory genes in a high resolution provides differences among strains. Therefore it is increasingly used as a taxonomic tool to replace DNA-DNA hybridization (Keto-Timonen *et al*., 2006).

Multi Locus Sequence Analysis was designated to provide an alternative approach for microbial taxonomy (Hanage *et al*., 2006). Given the robustness and discriminatory power of this method, it is becoming widely used as a reference method for population biology and epidemiological findings (Maiden *et al*., 2006). The method is based on the information that bacteria assigned to the same species should have protein-coding genes which are similar in sequence (Maiden *et al*., 1998). Thus it provides high discriminating potential for carefully selected and concatenated gene sequences (Gevers, 2005; Naser *et al*., 2005; Hanage, 2006; Kuhnert *et al*., 2006; Martens *et al*., 2008; Tailliez *et al*., 2010). The concatenated sequences of multiple housekeeping loci serve to detect the patterns of clustering among large populations of strains of closely related bacterial species. This approach provides more informative nucleotide sites than a single gene and consequently differentiates similar species and buffers against the distorting affects of recombination at one of the loci (Hanage *et al*., 2005a). Among DNA-sequencing methods, MLSA representing conserved genes and covering different regions of the genome is becoming widely used and accepted for differentiating taxa and establishing taxa boundaries (Maiden, 2006).

Generally new taxa are not accepted without comparison of 16S rRNA sequences to other related taxa. Based on rRNA, the tree of life can resolve the three domains of Archaea, Bacteria, and Eukaryotes. The 16S rRNA sequence has high value for determining the taxonomic position and phylogenetic relationships (Woese, 1987). A threshold of 97% homology is generally accepted as the cut-off value required for discriminating species. However, the differences in 16S rRNA sequences among taxa of the *Enterobacteriaceae* family are not discriminative enough to guarantee species identity as different studies reported very high similarity values (Fox *et al*., 1992; Tindall *et al*., 2010). The presence of multiple polymorphic copies of the 16S rRNA operon in the members of the family *Enterobacteriacae* makes the use of this genomic region problematic for taxonomic analysis. For example, *Pectobacterium carotovorum*, like other *Pectobacterium* species, encodes seven rRNA operons (Glasner *et al*., 2009).

Additional specific phenotypic characterization is needed before awarding the distinct genome species a separate name (Wayne *et al*., 1987; Brenner *et al*., 1982). In recent years a greater importance is being placed on the chemotaxonomic features, and it becomes a standard practice for taxonomic studies to include chemotaxonomic data in a novel species description (http://ijs.sgmjournals.org/site/misc/ifora.xhtml.). The chemotaxonomy refers to structural elements of the cell, cellular fatty acid composition, polar lipids, quinones and mycolic acid. The fatty acid's composition within members of the family *Enterobacteriaceae* is characteristic (Tindal *et al*., 2010). Taking into account that the phenotypic criteria and the chemotaxonomic criteria are the result of metabolic regulatory elements and characteristic genes, Brenner (1992) suggested that gene sequence approaches could replace the unique phenotypic or chemotaxonomic criteria generally needed to define a new taxon.

Validation a new species status is very critical in bacteria, requiring the proof of a wide range of characteristics, and the suggestion of a new name for a species or subspecies (Ad Hoc Committee on Approaches to Taxonomy within the *Proteobacteria*, Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematic) (Wayne *et al*., 1987; Murray *et al*., 1990). Additionally, different studies support infra divisions like infrasubspecies of bacteria to pave for more speciation and determination of bacterial groups (Bull *et al*., 2008). Polyphasic taxonomy based on consensus phenotypic, genotypic and phylogenetic characteristics provides a strong basis for delineating new taxa and validating suggested names. The choice of compatible methods to detect differences at the strain, species, genus or family level is decisive for achieving reliable results (Vandamme *et al*., 1996).

1.2. Nomenclature of Bacteria

To award a species name to a described taxon requires validation by the International Code of Nomenclature of Bacteria (1990) and Bacteriological Code (Washington, DC: American society for Microbiology), meanwhile publishing it in the International Journal of Systematic and Evolutionary Microbiology (formerly International Journal of Systematic Bacteriology). The naming process has to follow the standards approved by the previous committees, few conditions are listed, such as 'the generic and supergeneric names are single words, in Latin form, spelled with an initial capital letter,

the gender of specific or subspecific epithets must agree with each other and with the genus to which they belong (http://ijs.sgmjournals.org/site/misc/ifora.xhtml#policy-valid).

Bacteria are awarded a special interest from biologists in the field of nomenclature, and/or accurate description of the specific groups, their nomenclature is published in Bergey's manual of determinative bacteriology (1923), which provides identification schemes for identifying Bacteria and Archaea by the available morphology, differential staining and biochemical tests. As example, the family *Enterobacteriaceae* was accepted and described in the fifth edition of the Bergey's manual of determinative bacteriology (1939). More recently the Bergey's manual for systematic bacteriology (1980) provides phylogenetic information on Bacteria and Archaea based on rRNA sequencing and other available information. All validated bacterial families and genera are precisely described. The genus *Pectobacterium* belongs to the family *Enterobacteriaceae* which is a member of *Enterobacteriales*, *Proteobacteria*, *Gammaproteobacteria* in the kingdom of Bacteria.

As described in the last version of Bergy's manual, the *Enterobacteriaceae* are a family of Gram-negative, rod-shaped bacteria, non-sporeforming, facultative anaerobic with peritrichous flagellae, and includes human/animal and plant pathogens such as *Pantoea*, *Escherchia*, *Salmonella*, *Klebsiella*, and *Yersinia* and the organism under investigation, the soft rot *Erwinia* (pectolytic *Enterobacteria*).

1.3. The soft rot *Erwinia (Pectobacterium* genus)

The genus *Erwinia* was named after Erwin Frink Smith to include the plant pathogenic *Enterobacteria* in 1917. Since the establishment of this genus, many new genera have been split from *Erwinia* (Charkowski, 2006). In year 1945, a suggestion to separate the pectolytic *Enterobacter* into the genus *Pectobacteria* has been put forward by Waldee, (1945). The *Pectobacterium* naming was made based on the unique pectolytic activity of the bacteria, but the name faced unacceptability and was not in use (De Boer, 2003). Hauben *et al*., (1998) and Gardan *et al*., (2003) revived the name *Pectobacterium* and suggested it as the genus name harbouring strains belonging to the previous plant pathogen *E. carotovora* with its four subspecies, named in their epithets as *atroseptica*, *carotovora*, *betavasculorum* and *wasabiae*. The four subspecies were elevated to species status based on the 16S rRNA and DNA-DNA hybridization results of Hauben's and Gardan's studies, respectively, and the new six combinations *P. atrosepticum*, *P.*

carotovorum, *P. betavasculorum, P. wasabiae, P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *odoriferum* were given (Gardan *et al*., 2003). Unfortunately, the correct assigning of the soft rot pathogen to a separate genus and the switch between the *Erwinia* and the *Pectobacterium* genus name caused a considerable confusion in the literature.

All pathogens isolated from various hosts, such as arum, cabbage, carrot, celery, cotton, cucumber, maize, sugarcane, tobacco, dieffenbachia, potato and others were reduced to the species *E. carotovora* (Dye, 1969). Initially, the name '*Bacillus carotovorus*' (Jones, 1901) was created for strains isolated from carrot and other vegetables, later the name was changed to *E. carotovora* and finally *Pectobacterium carotovorum* and named as the type species of the genus (Waldee, 1945; Gardan *et al*., 2003). The '*Bacillus atrosepticus*' was created for the pathogen causing potato blackleg (van Hall, 1902), later changed to *E. atroseptica* and recently renamed *P. atrosepticum* (Hauben *et al*., 1999; Gardan *et al*., 2003). The *E. betavasculorum* was created for the pathogen associated with soft rot disease and vascular necrosis of sugar beet, recently renamed *P. betavascularum* (Thomson *et al*., 1981; Hauben *et al*., 1999; Gardan *et al*., 2003). The *E. wasabiae* originally was isolated from Japanese horseradish, but is causing soft rot disease of various vegetable, responsible for internal discoloration of rhizomes of wasabi, was recently renamed *P. wasabiae* (Goto and Motsomoto, 1987; Hauben *et al*., 1999; Gardan *et al*., 2003). Two subspecies were named under the species *P. carotovorum* of *P. carotovorum* subsp. *odoriferum* (Gallois *et al*., 1992; Hauben *et al*., 1999; Gardan *et al*., 2003) (formerly *E. carotovora* subsp. *odorifera*) was isolated from and associates with soft ort disease on witloof chicory, leeks, and celery, responsible for slimy odorous rot and *P. carotovorum* subsp. *carotovorum* (Jones, 1901) Hauben *et al*., 1999; Gardan *et al*., 2003) which reserved the original description of the species.

Commercially, *P. carotovorum* and *P. atrosepticum* were considered the most important soft rot pathogens within the *Pectobacterium* species. Both pathogens cause tissue maceration, termed soft rot disease by producing plant cell wall degrading enzymes (Toth *et al*., 2003). These two pathogens were recognized beforehand and differentiated by disease symptoms, host range, and the distribution regions. *P. atrosepticum* causes a vascular disease termed blackleg on potato plants in the field as well as a storage rot of potato tubers and has a limited host range almost exclusively to potato in temperate regions

while *P. carotovorum* causes mainly non-coloured decay, mainly in storage, on a wide variety of plant species, including potato, dominating in subtropical and temperated regions (De Boer, 2003).

For the purpose of identification the four *Pectobacterium* species and the two subspecies validated in the study of Gardan *et al*., (2003) which focused on the taxonomic criterion of DNA-DNA hybridization, several studies have been performed which yielded reliable phenotypic criteria that differentiate two or more species (Goto and Matsumoto, 1987; Thomson *et al*., 1981; Gallois *et al*., 1992). Serological differentiation methods were also under investigation and were found suitable for *P. atrosepticum* diagnosis, but not for *P. carotovorum* because of its high diversity (De Boer *et al*., 1979). Later the identification of the species was supported by molecular techniques, mainly a PCR based on the *pel* (pectate lyase) gene (Helias *et al*., 1998; De Boer and Ward 1995; Nassar *et al*., 1996), which discriminated strains belonging to the different species.

The natural relationship of *Pectobacterium* species was studied by analysis of 16S rRNA sequences (Kwon *et al*., 1997) revealing a deeper understanding of the heterogeneity and the intermixed among strains of the *Pectobacteria* and other *Erwinia* species. Hauben *et al*. (1998) studied the phylogeny based on the 16S rRNA sequence among the *Erwinia* members and confirmed three phylogenetically specific groups of *Pectobacterium*. Fessehaie *et al*. (2002) added additional sequenced data from the intergenic region (IGS), the IGS sequence data were failed to discriminate among the four species and revealed that strains belonging to one species fall in the phylogenetic group of another species. However, probable wrongly identified strains included in the study (Fessehaie *et al*., 2002) lead to lose the information of the sequence region under investigation. Avrova *et al*. (2001) used AFLP markers and defined three clusters corresponding to *Pectobacterium* species, cluster 1 harboring the *P. carotovorum* species with its two subspecies *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *odoriferum,* cluster 2 harboring strains belonging to *P. atrosepticum* and *P. betavasculorum*, and cluster 3 for *P. wasabiae* strains.

The extensive taxonomic investigations and nomenclature modification led to the situation that all not well characterized strains, which are not clearly assigned to one of the other three *Pectobacterium* taxa, fall in the fourth species *P. carotovorum* (Avrova *et al*., 2002; Gardan *et al*., 2003; De Boer, 2003). *P. carotovorum* have been described as a

heterogeneous species since it was reduced to one of four species in the *Pectobacterium* genus (Seo *et al*., 2002; Ma *et al*., 2007). Additionally, specific ecologically-adapted strains and unexpected variability among strains obtained from clonal hosts in one region and in one season (De Boer *et al*., 1979; Yap *et al*., 2004) were found within strains of *P. carotovorum.* The variation among the *P. carotovorum* strains could reflect the high diversity in gene regulatory mechanisms and their amenability to genetic exchange (Glasner *et al*., 2008).

The species *P. carotovorum* was described as the most diverse species in the *Pectobacterium* genus (Helias *et al*., 1998: Avrova *et al*., 2002: Ma *et al*., 2007). For *P. carotovorum* strains isolated from slimy rot of witloof chicory and designated as “atypical” *P. atrosepticum* strains - they produce reducing substances from sucrose and acid from α-methyl glucoside -, more investigation on DNA-DNA identity with other *Pectobacterium* species, carbon utilization patterns and other characteristics related to the ability of these strains to produce a typical bananalike odor when they were incubated onto chicory leaves, resulted in the designation of the strains to a new taxon under the *P. carotovorum* called *P. carotovorum* subsp. *odoriferum* (Gallois *et al*., 1992).

Other strains were described as “atypical” *P. atrosepticum* strains because of their ability to produce reducing substances from sucrose and acid from α-methyl glucoside, both characteristic were used as discriminative criteria to assign strains to the species *P. atrosepticum*. Strains associated with blackleg disease of potato were reported from Brazil with symptoms typical for *P. atrospticum* species (Duarte *et al*., 2004). The study suggested a new name to these strains: *E. carotovora* subsp. *brasiliensis.* Since the two studies of Duarte *et al*. (2004) and Gallois *et al*., (1992), who recorded atypical *P. atrosepticum* strains, the phenotypic criteria which were for a long term reliable to distinguish *P. carotovorum* and *P. atrosepticum* by production of reducing substances from sucrose and acid from α-methyl glucoside were no longer valid.

The *Erwinia carotovora* subsp. *brasiliensis* name was amended following the agreement to use the *Pectobacterium* genus name for pectolytic pathogens instead of *Erwinia* and the new combination *P. carotovorum* subsp. *brasiliensis* was in use. The *P. carotovorum* subsp. *brasiliensis* taxon was widely accepted by the scientific society to justify the heterogeneity of the species *P. carotovorum*. The subspecies of *P. carotovorum; P. carotovorum* subsp. *carotovorum and ‘P. carotovorum* subsp. *brasiliensis’* were

phylogenetically well distributed based on sequencing fragments of seven housekeeping genes from bacterial population of soft rot strains (Ma *et al*., 2007), confirming different genome designations for the three subspecies (Glasner *et al*., 2009).

Since the subspecies epithet *brasiliensis* was under discussion according to the reviewing process of the third chapter of the thesis, the name will be used as '*P. carotovorum* subsp. *brasiliensis'* in chapter I and II, following the first suggestion of the taxon by Duarte *et al*. (2004) and the microbiology code of invalid taxa. The name *P. carotovorum* subsp. *brasiliense* will be used in the rest of the thesis to confirm the new naming proved in the third chapter, following the requirement that the epithet has to follow the gender of the species and consequently the gender of the genus. According to the results of the third chapter the taxon will therefore have a legitimate name and does not need to be put between quotes.

An additional genus was split from the previous genus *Erwinia*, by transferring the bacterial plant pathogen species *E. chrysanthemi* (Burkholder *et al*., 1953) to the genus *Dickeya*. *E. chrysanthemi,* later called *Pectobacterium chrysanthemi* and was divided into pathovars and biovars (Toth *et al*., 2003). The more recent DNA-DNA hybridization, 16S rRNA gene sequences, RFLP data and phenotypic data (Nassar *et al*., 1996a; Samson *et al*., 2005) resulted in distribution of the former pathovars and biovars of the previous *P. chrysanthemi* in six genomic species as following: *Dickeya zeae*, *Dickeya chrysanthemi*, *Dickeya dieffenbachia*, *Dickeya dianthicola*, *Dickeya dadantii* and *Dickeya paradisiacal.* The *Dickeya* species mainly affect crops and other plants in tropical and subtropical regions and have a wide host range (Charkowski, 2006). Since there are no clear discriminating criteria which can assign strains to one of the six genome species, an MLSA approach is in preparation, which could help in grouping the bacteria in the appropriate genome species (Kowalewska *et al*., 2010). *D. solani* is a novel species, recently suggested in Europe as the causal agent of severe soft rot disease on potato (Toth *et al*., 2011).

The family *Enterobacteriaceae* is one of the most studied bacterial families because it contains the most investigated human and animal pathogens. Among plant pathogenic *Enterobacteriaceae*, *P. atrosepticum* SCRI 1043 was the first sequenced bacterium of 5,064,019 bp (GenBank accession number BX950851.1) (Bell *et al*., 2006). The genome of the plant pathogenic enterobacterium *P. atrosepticum* SCRI 1043 is similar in size to genomes of enterobacterial animal pathogens and shares with them common

enterobacterial genes. Approximately only 33% of the genome sequence of SCRI1043 is not shared with sequenced enterobacterial human pathogens (Bell *et al*., 2004; Toth, 2006).

Strain *Dickeya dadantii* 3937 was also sequenced as a bacterium of the *Pectobacterium* species before establishing the genus *Dickeya*. Later on a comparative genomic study of three *Pectobacterium* strains (Glasner *et al*., 2008) was established, namely *P. carotovorum* WPP14, *P. carotovorum* subsp. *brasiliensis* 212 and the sequenced bacterium *P. atrosepticum* SCRI1043. An account of 96% of the predicted genome size was used from both first and second strains in comparison to the whole genome sequence of the chromosome of the third strain. The venn diagram of the three drafted genomes illustrates a total amount of 95% orthologous proteins encoded by *P. carotovorum* and *P. carotovorum* subsp. *brasiliensis* identical between the two bacteria, and 92% identical to *P. atrosepticum.* Additional *Pectobacterium* strains were sequenced such as strain *P. carotovorum* Pc1 of 4,862,913 bp (GenBank accession number CP001657) and *P. wasabiae* 163 of 5,063,892 bp (GenBank accession number CP001790.1) by the US DOE Joint Genome Institute (JGI-PGF) and are in use for more comprehensive studies.

1.4. Geographical sources of the soft rot strains

Syria is a Mediterranean country in which potato (*Solanum tuberosum* L.) is widely cultivated as one of the major vegetable crops almost in all provinces and in three swing seasons. Planting and harvesting potato differ according to the season. Spring potatoes are planted from January to February, while harvesting of about 13 thousand hectares and 250 thousand tons starts in June. Autumn potato is planted from mid July to mid August, whereas harvesting of about 14 thousand hectares with a production of 240 thousand tones starts in early November and continues until the end of February. The area of summer potato is very limited, and does not exceed one thousand hectares in rural Damascus, where planting is conducted from April to mid May with a production of about 25 thousand tons (Alammouri, 2008).

The *Pectobacteria* are widespread in the environment and can live freely in an epiphytic or/and saprophytic lifestyles in soil, water and on the plant surface without causing disease (Perombelon and Kelman, 1980). Nevertheless, the *Pectobacteria* occurring in various ecological niches possess the key determinants of plant pathogens, including the production of highly destructive special plant cell wall degrading enzymes

which cause general tissue maceration (Toth, 2003). In some years we detected a severe infection of an *Enterobacter*-like plant pathogen which macerated and decayed potato plant tissue especially during harvesting the spring sowing potatoes, as well as in the storage. The best temperature for potato growth lies around 18-20 ^{o}C which also fits best to the development of *P. atrosepticum* potato blackleg pathogen. At higher temperature the plant has more stress and other soft rot pathogens such as *P. carotovorum* and *Dickeya* spp. are more likely to invade mostly injured plants.

For more than two years of survey covering most provinces where potato is grown in the different swing date summer, autumn and spring, we isolated pectolytic erwinias from most samples. The pectolytic efficiency of the strains was detected either by a pathogenicity test on ten different potato cultivars and/or by their ability to utilize pectin in crystal violet pectate medium (CVP). The strains were identified using conventional biochemical methods and proved to be belonging to the plant pathogens *E. carotovora* subsp. *carotovora, E. carotovora* subsp. *atrosepticum* and *E. chrysanthemi*. Some representative strains were stored under paraffin oil and considered for more comprehensive identification (Nabhan *et al*., 2006; Nabhan *et al*., 2009).

1.5. Objectives

Aim of the present study was to compare the pectolytic strains isolated from infected potato plants from Syria to reference strains collected from different geographical sources, hosts and at different times, and to determine their taxonomic identity and relatedness by molecular methods. These investigations support the recently emphasized needs for understanding the diversity of the bacterial plant pathogens belonging to the family *Enterobacteriaceae*. The study was carried out on 30 strains from Syria and 46 strains collected from international microbial collections and laboratories, representing mainly the genus *Pectobacterium*, and the genus *Dickeya* for comparison. Objectives and achievements in detail were:

- Investigate the genomic designation and characterization of 63 strains from the collected population by epidemiologic, genetic and phylogenetic studies
  In chapter II, the study used the most comprehensive methods to consider population genetics and resolved the insufficient criteria provided by classical phenotypic classification methods which lead to inter-species mixing. Both methods used, MLSA and AFLP, revealed complementary results and served as basis for the studies in chapters II, III and IV. The study re-identified the strains and revealed the highest number of strain clustering in the phylogentic cluster of the *P. carotovorum* species with its four subclusters.
- Determine the taxonomic relationships among four *P. carotovorum* clusters resolved in chapter II.
  In chapter III, the study utilized the strains clustered in *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum* and *P. carotovorum* subsp. *brasiliensis* phylogenetic clades. Additional eight strains from Canada and each of the type strains of *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *odoriferum* were added to the strain collection. The research in chapter III investigated all properties needed to validate the taxon ‘*P. carotovorum* subsp. *brasiliense* nov. subsp.’ such as DNA-DNA association, natural relationships among the three *P. carotovorum* subspecies based on 16S rRNA sequence data, phenotypic criteria, GC % mol content of the suggested type strain and MLSA data.

- Verify the status of the new phylogenetic clade within the *P. carotovorum* clusters generated in chapter II

  In chapter IV the phylogenetic clade within the *P. carotovorum* clusters was studied in the light of its relationship with the other four *Pectobacterium* species and the three *P. carotovorum* subspecies. Type strains of each *P. atrosepticum*, *P. wasabiae* and *P. betavasculorum* were added to the study. The investigated cluster harbors strains mainly obtained from monocotyledones and two dicotyledones plants and has a similar genome designation based on the AFLP banding patterns generated in chapter II. The chapter IV added an additional DNA-DNA association study, *in-silico* DNA-DNA association approach, natural relationships among the seven *Pectobacterium* taxa based on 16S rRNA gene, phenotypic characteristics, GC % mol content, pathogenicity test, RFLP study and a comprehensive historical revision of the idle *E. aroideae* (1904) taxon. The study recovered the epithet '*aroideae'* and established the new fifth *P. aroideae* species of the *Pectobacterium* genus.

In total a number of 608 gene sequences and 64 16S rRNA homologs throughout the thesis [Table S3 (3.1, 3.2, 3.3)], in addition to GenBank sequence data of 280 sequences (Table S1) served to pursue the objectives of this study. All new strains integrated in this work were deposited in the French collection of plant pathogenic bacteria (CFBP), and eight of them additionally deposited in the German resource centre for biological material (DSMZ) (Table S6).

2. The use of two complementary DNA assays, AFLP and MLSA, for epidemic and phylogenetic studies of pectolytic enterobacterial strains with focus on the heterogeneous species *P. carotovorum* [1]

2.1. Abstract

Amplified Fragment Length Polymorphism (AFLP) markers and Multi-Locus Sequence Analysis (MLSA) were used to analyze 63 bacterial strains, including 30 soft rot-causing bacterial strains collected from Syrian potato fields and 33 reference strains. For the MLSA, additional sequences of 41 strains of *Pectobacterium* and *Dickeya*, available from the NCBI GenBank, were included to produce a single alignment of the 104 taxa for the seven concatenated genes (*acnA*, *gapA*, *proA*, *icd*, *mtlD*, *mdh*, *pgi*). The results indicate the need for a revision of the previously classified strains, as some potato-derived *P. carotovorum* strains were re-identified as *P. wasabiae*. The strains that were classified as *P. carotovorum* during the analyses demonstrated high heterogeneity and grouped into five *P. carotovorum* highly supported clusters (PcI to PcV).

The strains represented a wide range of host plants including potatoes, cabbage, avocado pears, arum lilies, sugar cane and more. Host specificity was detected in PcV, in which four of the six strains were isolated from monocotyledonous plants. The PcV strains formed a clearly distinct group in all the constructed phylogenetic trees. The number of strains phylogenetically classified as subspecies '*P. carotovorum* subsp. *brasiliensis*' in PcIV dramatically increased in size due to the characterization of new isolates or re-identification of previous *P. carotovorum* and *P. atrosepticum* strains. The *P. carotovorum* strains from Syria were grouped into PcI, PcII and PcIV. This grouping indicates a lack of correlation between the geographical origin and classification of these pathogens.

2.2. Introduction

The pectolytic grouping of the Enterobacteriaceae family (formerly the *Erwinia* soft-rot group) is comprised of the genera *Pectobacterium* (*E. carotovora*) and *Dickeya* (*E. chrysanthemi*). The bacteria from these two genera have been classified as two separate soft-rot causing pathogens; however, they do share some common biochemical and genetic characteristics (Dickey, 1979).

[1] Plant Pathology. Doi: 10.1111/j.1365-3059.2011.02546.x

The results of DNA-DNA hybridization experiments helped reclassify *Pectobacterium chrysanthemi* into the genus *Dickeya* (Burkholder *et al*., 1953), where it was subsequently divided into five genetic species missing until now diagnostic determination tests (Samson *et al*., 2005). According to Gardan *et al*. (2003) the former species *E. carotovora* was incorporated into the genus *Pectobacterium,* and four of its established subspecies were renamed as *P. carotovorum*, *P. atrosepticum*, *P. betavasculorum,* and *P. wasabiae*. None of the last three species were further subdivided into subspecies because of a low level of intraspecific genetic diversity (Avrova *et al*., 2002).

The species *Pectobacterium carotovorum* is comprised of different variable strains, a small number of which are grouped under the *P. carotovorum* subsp. *odoriferum* (Gallois *et al*., 1992) some under '*P. carotovorum* subsp. *brasiliensis*' (Duarte *et al*., 2004) and yet more strains under *P. carotovorum* subsp. *carotovorum*. Both *P. carotovorum* subsp. *odoriferum* and '*P. carotovorum* subsp. *brasiliensis*' are described as atypical *P. carotovorum* strains because of their biochemical characteristics.

A potential classification problem arises from non-clustered *P. carotovorum* strains or strains with non-standard biochemical or genetic characteristics. Such deviations reduce the overall utility of using biochemical studies to characterize isolates of these pathogens. Moreover, there is significant genomic heterogeneity among the *P. carotovorum* strains classified by DNA-DNA hybridization (Gardan *et al*., 2003) further indicating that taxonomy of this highly divergent species is problematic.

The wide host range of *P. carotovorum,* which covers more than 35% of all angiosperm plant orders (Ma *et al*., 2007) and the ability of the *P. carotovorum* strains to survive in different environments (Toth *et al*., 2003) is the probable reason for the high genetic diversity of the pathogen. Therefore, a clear taxonomical description of this group is vital to understanding the pathogenic differences and geographical adaptations of the species (Glasner *et al*., 2008).

Among the molecular methods used for classification, examination of amplified fragment length polymorphisms (AFLPs) is a technique used for genomic identification and delineation of bacterial plant and human pathogens. As AFLPs target multiple coding and non-coding loci in the genome, the technique is widely used (Portier *et al*., 2006). Avrova *et al*. (2002) successfully used AFLPs to analyze *Pectobacterium* strains and

discovered extreme diversity within *P. carotovorum*. Classification studies based on Multi Locus Sequencing Analysis (MLSA) may provide phylogenetically relevant sequence information that can aid in a better understanding of speciation events in bacteria (Hanage *et al*., 2006). The results of previous analyses (Kim *et al*. 2009) indicate that MLSA is one of the most reliable methods to differentiate *P. carotovorum* strains.

The pectolytic bacteria are a group of economically very important plant pathogens on potatoes in Syria. Potatoes are grown in three sowing seasons in Syria and the production losses caused by pectolytic bacteria ranges from 1-2% up to 50% mainly based on the high variation in irrigation conditions in different fields (Nabhan *et al*., 2009). In Syria, severe symptoms occur mainly in the hot spring and summer sowing season were irrigation of the fields is often needed and rarely in the cooler autumn sowing season. The pathogens *P. carotovorum*, *P. atrosepticum* and *Dickeya* spp. are the main cause of soft rot and blackleg disease on potato in Syria (Abu-Ghorrah *et al*., 2000; Nabhan *et al*., 2006). Evaluating ten locally grown potato cultivars against pectolytic bacterial strain population isolated from potato fields in Syria showed that some cultivars like Draga, Diamant and Anna were significantly more resistant in comparision to the cultivars Arenda, Lezetta and Nicola torwards these local strains.

In this chapter, we aimed to elucidate the population structures of pectolytic enterobacterial strains with an emphasis on *P. carotovorum* strains and to determine the genetic diversity of strains sampled in Syria using AFLPs and MLSA.

2.3. Materials and Methods

Two groups of strains were included in this study of 63 strains (Table 1). Among them, 33 reference strains of *Pectobacterium* and *Dickeya* species and a collection of 30 pectolytic enterobacterial strains, which were isolated from infected potato plants sampled in 2002-2004 from different fields in Syria. The strains were biochemically characterized (Schaad, 1988), including characterization of their ability to utilize pectin on Crystal Violet Pectate (CVP) medium. They were also analyzed by PCR using primers Y1, Y2 to identify *P. carotovorum* strains, primers ECA1f, ECA2r specific for *P. atrosepticum*, and ADE1, ADE2 primers specific for *Dickeya* (Darras *et al*., 1994; De Boer & Ward 1995; Nassar *et al*., 1996).

Bacterial cultures

Cultures of strains were obtained by inoculation of single colonies and stored in 40% glycerol at -80°C and in CRYOBANK^{TM} (MSAT, UK). In addition, the strains were routinely plated on King's B agar medium

Table 1. Soft-rot enterobacterial strains included in this study

	Bacterial strain	Host Plant	Location	Source
	Pectobacterium carotovorum			
1.	4.3.14	*Aloe arborescens/cactus*	Germany	JKI
2.	2 (NCPPB275) +	*Solanum tuberosum*	USA	SCRI
3.	4.3.8	*Brassica oleracea*		JKI
4.	121 (NCPPB1640) ^{X}	*Saccharum* spp./Sugar cane	Jamaica	SCRI
5.	109 (NCPPB929) ^{X}	*Zantedeschia aethiopica* /Arum lily	South Africa	SCRI
6.	3 (NCPPB435) + ^{X}	*S. tuberosum* (Potato stem)	Zimbabwe	SCRI
7.	102 (NCPPB547)^{X}	*Persea americana* (Avocado pear)	Israel	SCRI
29.	A6.2, A10.1, A10.2, A16, A18, C3, C137, C140.2, C142.1b, C142.1s, C142.2 C143, C144, C150, C267, C331, C338, C364.2, C380, C412.4, M30, N78	*S. tuberosum* (2002-2004)	Syria	This study
	'*Pectobacterium carotovorum* subsp. *brasiliensis*'			
30.	4.3.22 (BBA J594)	*S. tuberosum*	Germany	JKI
31.	132	*Daucus carota* subsp. *sativus*	Japan	SCRI
32.	1073 +	*S. tuberosum*	Peru	SCRI
37.	A17, C18, A45, C317.1, C393.1	*S. tuberosum* (2002-2004)	Syria	This study
	Pectobacterium carotovorum* subsp. *odoriferum			
38.	NB1892			
39.	568 *	*Apium graveolens* 1983	Switzerland	JKI
40.	582 *	*Cichorium endivia* 1985	Switzerland	JKI
	Pectobacterium atrosepticum			
41.	M37	*S. tuberosum*	Syria	This study
42.	1071 (NCPPB 549)	*S. tuberosum*	UK	SCRI
43.	8	*S. tuberosum*	Netherland	SCRI
44.	185	*S. tuberosum*		JKI
45.	17A-1	*S. tuberosum*	Poland	JKI
46.	4.2.9	*S. tuberosum*		JKI
47.	4.2.5	*S. tuberosum*		JKI
	Pectobacterium wasabiae			
48.	1A-1 *	*S. tuberosum*	Poland	JKI
49.	207 (NCPPB1274) *	*S. tuberosum* 1962	Ireland	SCRI
50.	4.2.6 (St. 95) *	*S. tuberosum* 1989	Germany	JKI
	Pectobacterium betavasculorum			
51.	NB 2122	*Beta vulgaris*		
	***Dickeya* spp.**			

52.	4.9.3 (A15)	*Ipomoea batata*	USA	JKI
53.	4.9.4 (NCPPB1065)	*Zea mays* 1961	Egypt	JKI
54.	4.9.22			JKI
55.	SR260 (R.Montgomery)			JKI
56.	4610	*Chrysanthemum morifolium* 1956	USA	GSPB
57.	30177	*Chrysanthemum* sp. 1953	USA	GSPB
58.	30178	*Palaenopsis* sp. 1953		GSPB
59.	30179	*Saintpaulia ionantha*	Germany	GSPB
60.	3937 (CFBP3855)	*Saintpaulia ionantha*	France	JKI
61.	436*	*S. tuberosum*		GSPB
63.	C89.1, C89.2	*S. tuberosum*	Syria	This study

* received as *P. carotovorum* subsp. *carotovorum*
+ received as *P. atrosepticum*
^{X} expected to belong to unknown species
Of the 33 reference strains, 14 strains were received as *P. carotovorum* subsp. *carotovorum* under the former name (*Ecc*), *Ecc*436 strain was re-identified as *Dickeya* spp., *Ecc*1A-1, Ecc207, and *Ecc*4.2.6 strains were re-identified as *P. wasabiae, Ecc*568 and *Ecc*582 strains were re-identified as *P. carotovorum* subsp. *odoriferum*, *Ecc*132 and *Ecc*4.3.22 strains were re-identified as *P. carotovorum* subsp. *brasiliensis*.
Nine strains were received as *P. atrosepticum* under the previous name (*Eca*), *Eca*1073 strain was re-identified as *P. carotovorum* subsp. *brasiliensis*, *Eca*2 and *Eca*3 were re-identified as *P. carotovorum*.

NCBI GenBank electronic data

In addition to the 30 strains sampled in Syria and the 33 reference strains (Table 1), publically available electronic of sequenced housekeeping genes (Ma *et al*., 2007) from 40 strains of *Pectobacterium* and *Dickeya* were retrieved from the NCBI GenBank (Table S1). The sequences of (*acnA*, *gapA*, *proA*, *icd*, *mtlD*, *mdh*, *pgi*) housekeeping genes of the strain *P. carotovorum P.carotovorum*1 (accession number CP001657) were also obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank/index.html). Including the GenBank data, 104 taxa were subjected to MLSA.

DNA extraction

A single bacterial colony was inoculated into 20 ml of liquid Luria Broth medium and incubated overnight in a shaking incubator set at 27°C and 120 rpm. The bacteria cells were harvested from 1 ml of the 16 h-old LB cultures at a density of about $2x10^9$ CFU/ml. The DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen, Göttingen, Germany), following the instructions as described by the manufacturer. The extracted DNA was quantified on 1% agarose gels, with λ DNA serving as a reference ladder. The gels were examined with a Gel-Pro-Analyzer (INTAS-Germany) and by photometry at 260 and 280 nm. All DNA samples were stored at -20°C until used.

AFLP analyses

AFLP analyses were performed as described by Malek *et al*. (2000), with minor modifications. First, 200 ng of DNA from each strain was digested with restriction endonucleases and ligated. All preamplification reactions were conducted with primers lacking any selective nucleotides. The selective amplifications were made with 9 primer combinations. We used a combination of IRD 700 end-labeled *Hind*III primers with no selective nucleotide (MWG Biotech, Ebersberg, Germany) and unlabeled *Mse*I plus two selective nucleotides (AA, AC, AG, CC, GA, GG, TC, TG and TT). The PCR-products were separated on 6% polyacrylamide gels (Sequagel XR) and analyzed on a Licor-DNA-Analyzer, Gene ReadIR 4300 (MWG Biotech). The reproducibility of the strain's banding profiles was tested and confirmed by separate AFLP preamplification and end reactions from each of two independent DNA isolations. Ten arbitrarily selected strains were used for the reproducibility tests. Independent repeat reactions were run on the gels at different time points. The numerical analyses of the binary data were conducted in the FAMD 123 software (Schlüter and Harris, 2006), using the Jaccard similarity coefficient for computing genetic similarities. The dendrograms were constructed as UPGMA majority-rule consensus trees after 1000 bootstrap replicates.

MLSA analyses

Eight informative housekeeping genes were used in the multilocus sequence analysis. Following the study of Ma *et al*. (2007), fragments of the seven metabolic genes; aconiate hydrase 1 (*acnA*), glyceraldehyde-3-phosphate dehydrogenase A (*gapA*), isocitrate dehydrogenase (*icdA*), malate dehydrogenase (*mdh*), glucose-6-phosphate isomerase (*pgi*), mannitol-1-phosphate 5-dehydrogenase (*mtlD*), γ-glutamylphosphate reductase (*proA*), and the RNA polymerase subunit sigma factor 38 (*rpoS*) (Waleron *et al*., 2008) were amplified using degenerated primers. The *Pgi* gene was amplified using primer pair 815/396 for all *P. carotovorum* and *P. atrosepticum*, and using primer pair F2/R2 for *P. wasabiae*.

The PCR reactions were carried out using 50 ng DNA, 1x GeneAmp PCR buffer, 1 mM $MgCl_2$, 200 mM each dNTP (Fermentas, Germany), 10 pmole of each primer and 1.0 U of *Taq* DNA polymerase ($2.2x10^{-5}$ errors per nucleotide per cycle) (Fermentas, Germany) in a total volume of 25 µl. The PCR amplification program consisted of an initial denaturation at 94 °C for 4 min followed by 30 cycles at 94 °C for 30 s, annealing at

52 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min in a Biometra T-gradient thermocycler. The PCR products were separated on 1% agarose gels in 1x TAE buffer at 100 V for 45 min. The PCR products were purified from the agarose gel using a Gel-Extraction-Kit (Seqlab-Germany). Sequencing of the samples was performed by Seqlab (Göttingen, Germany) using the extended Hot-Shot method [BigDye Terminator ready reaction mix v3.1 (ABI)].

Phylogenetic analyses of the housekeeping genes

Multiple alignments were generated for the individual sequences of *acnA*, *gapA*, *icdA*, *mdh*, *pgi*, *mtlD*, *proA* and *rpoS*, using clustal W in Mega version 4.0 (Tamura *et al*., 2007) with a gaps penalty of 15. Phylogenetic and molecular evolutionary analyses were conducted in Mega 4.0. Three different parameters were used to compute maximum parsimony: consensus trees, neighbor joining trees and minimum evolution trees for each of the sequence data sets. Two nucleotide models of Maximum Composite Likelihood (McL) (Tamura & Nei, 1993) and p-distances (Nei & Kumar, 2000) were implemented for the sequences for the seven genes of the 104 taxa with bootstrapping test of 5000 replication.

Following that, the gene sequence data sets were analyzed to determine the appropriate model of evolution using FindModel http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html. The best resulting models were as follows: general time reversible plus gamma (GTR+G) (for gamma distribution see Tamura *et al.* (2004) for the *pgi* and *proA* sequence data sets, equal-frequency Tamura-Nei plus gamma (TrNef+G) for the *icdA* sequence data set, equal-frequency transition model plus gamma (TIMef+G) for the *mdh* sequence data set, symmetrical model plus gamma (SYM+G) for the *rpoS*, *gapA* and *acnA* data sets and the transition model plus gamma (TIM+G) for the *mtlD* data set.

The parameters for each model (Posada and Crandall, 2001) were implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2003; Ronquist and Huelsenbeck, 2001) (Table 2) and used to construct Maximum likelihood (ML) trees. For data more than 20000 bp, or concatenated data, the selection of the best-fit model of nucleotide substitution was conducted in Mega 5 (Tamura *et al*., 2007), and the ML trees were constructed in PhyMl (Guindon and Gascuel, 2003).

Table 2. Parameters of evolutionary models computed for each sequence data set and implemented in Mr. Bayes.

Sequence data set	No. taxa	No. char	AIC	*-Lnl*	Ts/Tv	G shape
icdA	104	479	2320.1	1157.0	4.43	0.26
acnA	104	275	3859.5	1923.8	1.72	0.32
Pgi	103	492	6453.4	3217.7	4.56	0.23
proA	104	614	12658.4	6320.2	3.43	0.30
gapA	103	408	3960.4	1974.2	1.72	0.17
mtlD	104	269	3256.2	1621.1	2.97	0.36
Mdh	104	437	4966.9	2519.4	3.36	0.22
Concatenated seven gene portions	104	2973	33510.3	16538.9	2.84	0.46
rpoS	63	779	4966.9	2477.4	3.15	0.43
Concatenated eight gene portions	63	3754	45488.8	22613.3	2.68	0.57

No. char: Number of characters
-lnL: Maximum Likelihood value.
f: base frequencies (0.25)
Ts/Tv estimated values of Transition/Transversion
G shape: Gamma distribution

Pathogenicity assays

Out of the 63 strains, the 28 *Pectobacterium* and the two *Dickeya* strains isolated from Syria, were subjected to a pathogenicity test on tomato plants (*Lycopersicum esculentum*) genotype L390. The thirty strain were tested by both stem and soil inoculation (Dannon and Wydra 2004; Costa *et al*., 2006) on the tomato L390, seven plants per strain and the experiment repeated minimal of two times for each strain. The method was adjusted using 25 days old tomato plants. Twofold stem inoculation were performed using 10 µl in each inoculation point with $2.4*10^8$ CFU ml^{-1} bacterial suspensions of 24h old bacterial cells. Inoculation was performed by injection into the plant stem at the third and fourth axial leaf from the base using HAMILTON syringe. Disease severity were scored as [0, 1, 2, 3, 4, 5] over 3 time points in two days interval. Moreover the pathogenicity of the strains were tested against tomato plant by soil inoculation, soil was inoculated using 0.1 ml bacterial suspension for each 1g soil substrate.

2.4. Results

AFLP analyses

AFLP reactions using nine primer combinations produced 925 clearly scorable DNA-fragments. The phylogenetic tree (Figure 1) derived from the AFLP analysis

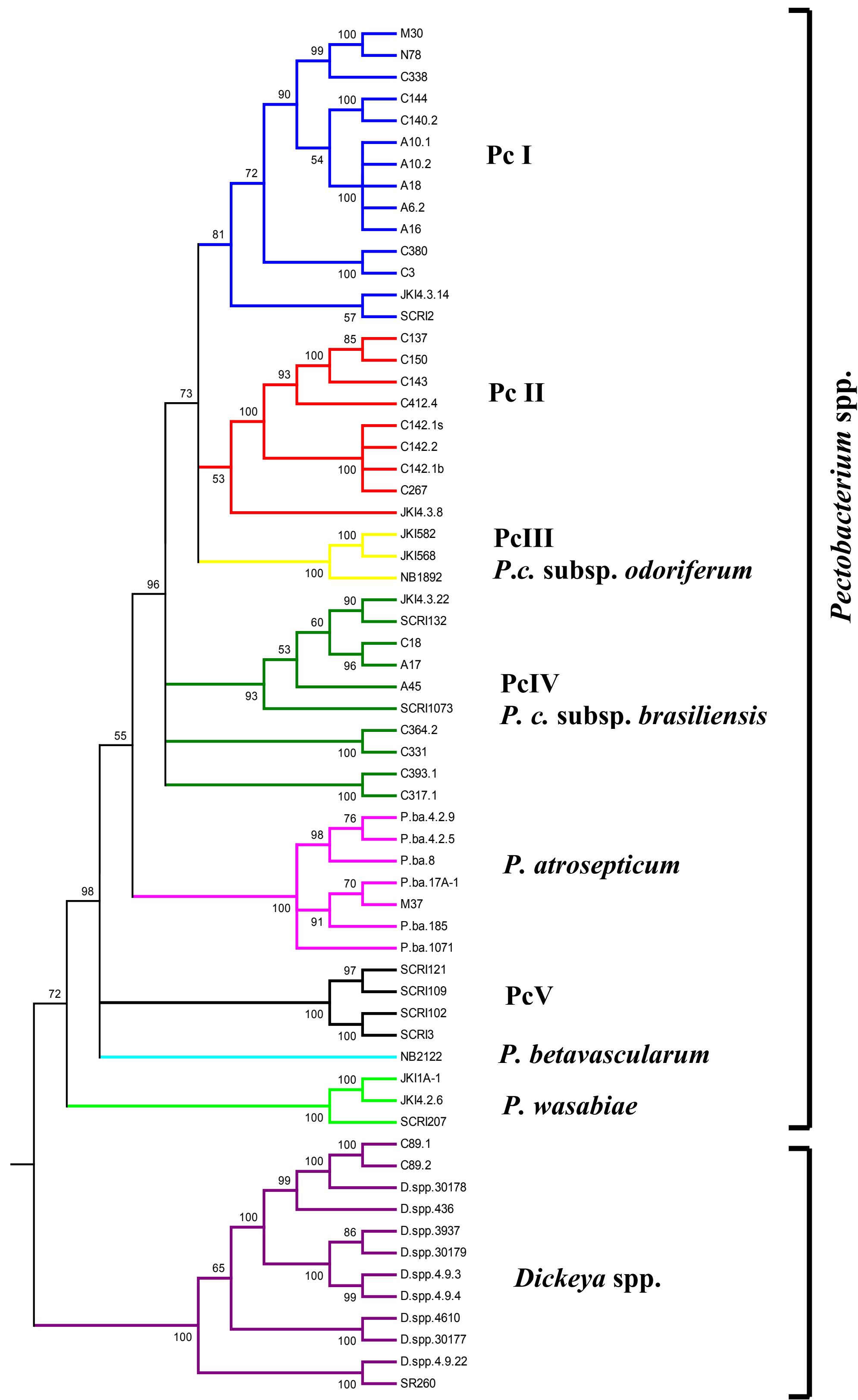
delineated 63 strains of *Pectobacterium* and *Dickeya*. The *Pectobacterium* species showed diverse banding profiles and clustered separately from the strains of *Dickeya*. Seven strains confirmed by PCR as *P. atrosepticum* grouped into one distinct group. Three *P. carotovorum* strains isolated from potatoes also separately clustered into one group. These strains were later identified as *P. wasabiae* by MLSA. Thirty-six of the forty strains of *P. carotovorum* formed a large group that subdivided into four subgroups (PcI to PcIV), whereas one additional subgroup (PcV), together with one strain of *P. betavasculorum* clustered between, *P. atrosepticum* and *P. wasabiae*. The *Dickeya* spp. (12 strains), including two strains from Syria (C89.1 and C89.2), formed one cluster distinct from all other analyzed *Pectobacterium* strains. The cluster was further subdivided into four sub-groups.

MLSA analyses of the housekeeping genes

Analysis of all 63 strains indicated that there were eight conserved genes across all strains. The analysis of the eight conserved genes generated of 501 (Table S3) characterized sequences. The sequences were submitted to the NCBI GenBank under accession numbers of HM156760 to HM157253 (http://www.ncbi.nlm.nih.gov/). A MLSA Maximum likelihood (ML) dendrogram was computed from concatenated sequences obtained from eight genes of each of the 63 taxa based on the GTR+G+I model (Figure S1). To analyze our samples within a broader range of germplasm, we obtained 40 additional sequences from NCBI GenBank, as well as information from the fully sequenced genome of *P. carotovorum* (strain Pc1) and reanalyzed, in the context of this extended dataset, seven of the eight genes examined in our first MLSA analysis. The maximum likelihood (ML) tree for this dataset (Figure 2) was constructed based on the substitution model TN93 being applied to the whole concatenated sequence. This extended set of genotypes represents a wider geographic distribution and a wider host range than our original set, and therefore may provide a better resolution of the taxonomic relationships within the subspecies of *Pectobacterium*. The phylogenetic relatedness found between ML trees constructed from the 63-strain/8-gene and 104-strain/7-gene MLSA analyses indicated a very good correlation (Figures S 1 and Figure 2). The overall genetic diversity in both trees was about 9.7%, suggesting that the value is the average genetic diversity in the conserved genes of these pathogens.

Figure 1

Majority rule consensus tree of 63 strains from genera *Pectobacterium* and *Dickeya* based on 925 AFLP fragments. The evolutionary relationships of the 63 taxa were inferred using the UPGMA method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches.



All values below 50 were cut off.

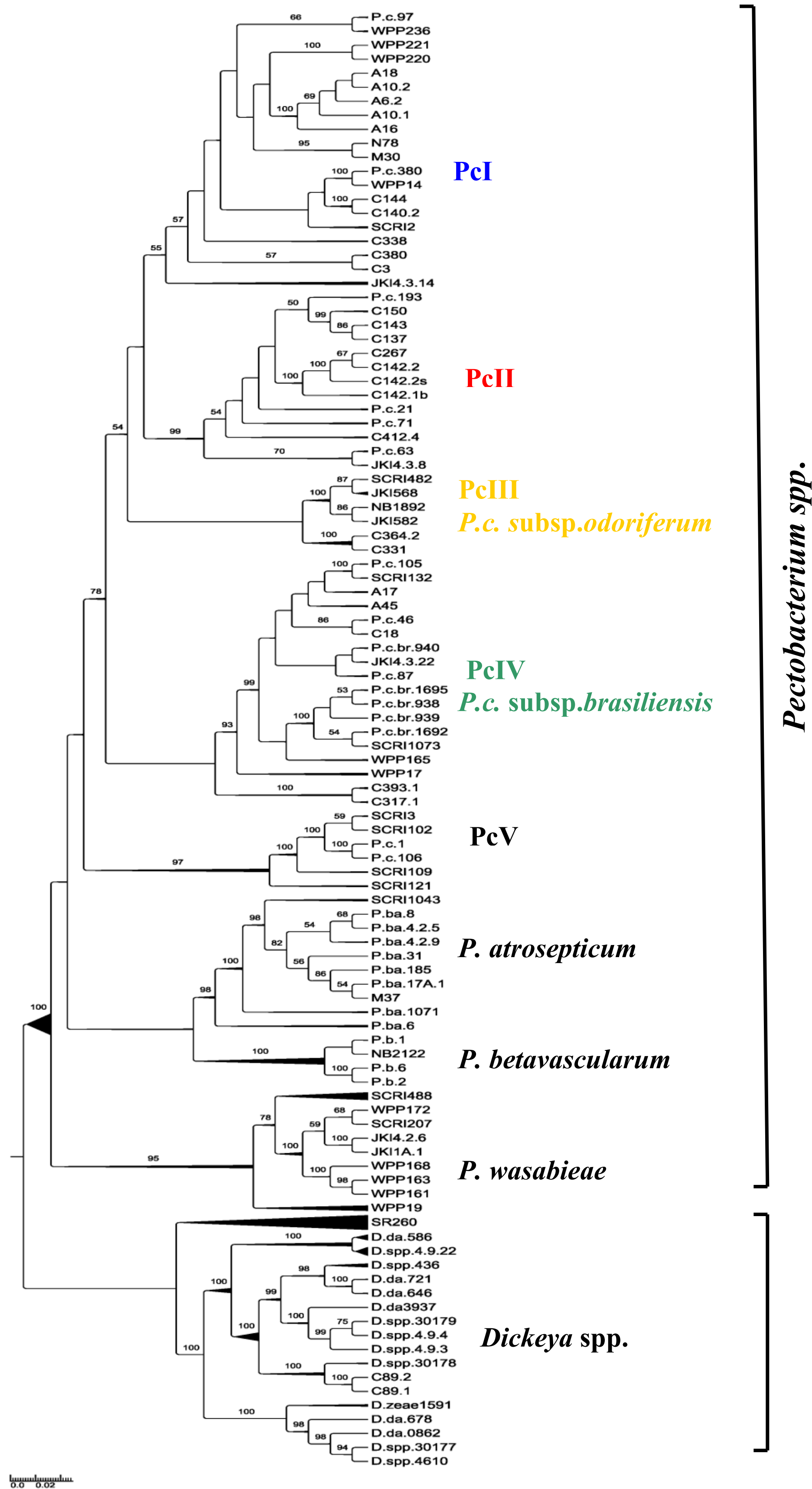
Although AFLP markers provide high information content and lead to highly reliable tree topologies, an assignment, by AFLP to a particular species is difficult without reference strains being included in the AFLP gels. However, a differentiation at the species level can be achieved by using DNA sequences of published reference strains as exemplified by the three *P. carotovorum* strains (SCRI 207, JKI 1A-1, JKI 4.2.6) which re-identified as *P. wasabiae*. Therefore, using AFLP corroborates the utility of MLSA as a classification method for poorly characterized bacteria such as *Pectobacterium*. Another advantage of sequenced-based analyses is the possibility of computing a separate dendrogram for each individual gene of interest to analyze gene flow. If the exchange of genetic material between genetically defined groups of taxa occurs, this should be reflected by divergent topologies in the single-gene trees. The ML dendrograms computed separately for the genes *acnA*, *gapA*, *icdA*, *mdh*, *mtlD*, *pgi*, *rpoS* and *proA* indicated the rare appearance of single strains within the cluster of a different species (Table S2, Figures S2 to 9). This occurrence was observed for two out of the eight analyzed genes in strains *P. atrosepticum Pba*6 and *P. carotovorum* SCRI121. Therefore, these strains must be regarded as more polymorphic than other strains of the same species.

A gene-dependent clustering was observed for the strain *Dickeya* spp. 436. The strain clusters into the *P. carotovorum* subsp. *odoriferum* group if only the *icd* gene is considered, clusters into the *P. atrosepticum* group for the *rpoS* gene, but is correctly placed into the *Dickeya* group by the MLSA approach. This *Dickeya* strain displays similarity to strains of *Pectobacterium* (Table S2). The similarity may explain the strain's previous characterization as a *Pectobacterium* strain, which was changed following PCR-based diagnostics (Nassar *et al*., 1996). As these single gene-dependent clusters are supported by individual bootstrap values larger than 50%, they are likely not the result of insufficient data but more likely illustrate horizontal gene flow and subsequent recombination events occurring between strains from different species. Although different mechanisms are involved in gene transfer, conjugation is considered to be the most widely occurring mechanism for gene flow between gram negative bacteria.

Figure 2

Maximum likelihood dendrogram generated using seven concatenated housekeeping gene sequences representing the evolutionary relationships among 86 strains of *Pectobacterium* spp. and 18 strains of *Dickeya* spp. The evolutionary relationships of the 104 taxa (63 taxa from our study and 41 taxa retrieved as electronic data from NCBI GenBank) were inferred using the Maximum likelihood method. The percentage of the replicate trees in which the

associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches.



All values below 50 were cut off.

2.5. Discussion

The MLSA tree topology of each of the eight and seven concatenated genes matches the dendrogram obtained by the AFLP analyses with one exception of the PcV cluster. These results are in agreement with other published studies using this combination of methods (Ngoc *et al*., 2010).

The data generated for the 104 datasets revealed an average molecular diversity of 6% among *Pectobacterium* strains, which formed groups clustering with the same topology as those published by Ma *et al*. (2007). Strains form the following four groups: the *P. atrosepticum* group (10 strains), the *P. wasabiae* group (9 strains), the *P. betavasculorum* group (4 strains) and a pooled *P. carotovorum* group of 63 strains in which all other strains are clustered. Genetic diversities of 0.01% and 1% were observed within the strains of each of *P. betavasculorum* and *P. atrosepticum*, respectively. A high level of homogeneity in *P. atrosepticum* has also been demonstrated in other studies (Darras *et al*., 1994; De Boer & Ward, 1995; Persson *et al*., 1995). *P. carotovorum,* comprising five subclusters (PcI-PcV), is considered to be more closely related to *P. atrosepticum* than *P. betavasculorum* or *P. wasabiae,* each with average genetic diversities of 7.3, 8 and 8.6%, respectively (Avrova *et al*., 2002).

The *P. wasabiae* group (Goto & Matsumoto, 1987), with a genetic diversity of 4.6%, is comprised of one strain isolated from Japanese horseradish and 8 strains isolated from potatoes. *P. wasabiae* was recently isolated from potatoes (Ma *et al*. 2007; Pitman *et al*., 2009). The presence of the species on potatoes does not reflect a recent extension of the host range for this taxon because some former *P. carotovorum* strains isolated from potatoes were subsequently re-identified as *P. wasabiae* in this study.

The topology of the trees obtained from both AFLP analysis and MLSA revealed that *P. carotovorum* is a highly diverse plant pathogen and comprises five genetic clusters (PcI to PcV). Previous studies based on either AFLP or MLSA placed *P. carotovorum* strains in a maximum of three different subclusters (Avrova *et al*., 2002). More recent studies support the existence of additional groups of genotypes. This group expansion relies on the identification of strains from monocotyledonous plant families based on both genetic and pathogenicity data (Yishay *et al*., 2008).

P. carotovorum strains isolated from Syria, with an average diversity of 3.6%, are distributed over the PcI, PcII and '*P. carotovorum* subsp. *brasiliense*'*s* groupings. The

Syrian strains did not form a monophyletic cluster, even when the growing season, the province and the year of isolation was considered. These results are similar to results by Yap *et al*. (2004), who observed unexpected variability between strains obtained from the similar environmental conditions. In addition, no monophyletic cluster was detected for the 45 strains isolated world-wide from potatoes. The potato strains grouped into four of the five *P. carotovorum* clusters with a genetic diversity of 4.0% within the strains showing potato as a common host.

Average diversity of 4.2% among all 63 strains of *P. carotovorum,* irrespective of host, comparing with 4.0% diversity among *P. carotovorum* strains isolated only from potato also demonstrated that strains that can infect any other hosts can easily infect potatoes (Toth *et al*., 2003; Glasner *et al*., 2008), with the exception of *P. carotovorum* subsp. *odoriferum* strains. *P. carotovorum* combines divergent strains clustered separately in five genetic clusters. These clusters, PcI, PcII, PcIII, PcIV and PcV, are situated equally at a genetic distance of 8% from the *P. betavasculorum* and *P. wasabiae* groups and at genetic distances of 7, 8, 8, 7 and 8% from *P. atrosepticum,* respectively.

The 20 strains within PcI, isolated from different geographical regions and various plant hosts (e.g., potato, marigold, cabbage, burdock, and aloe arborescens), displayed a high genetic diversity of 2.5% between strains and distances of 3.7-6.5% to the four other *P. carotovorum* clusters.

In PcII (13 strains), all strains were isolated from potato plants with the exception of strain JKI 4.3.8 isolated from *Brassica oleracea*. However, the strains in PcII were collected from different geographical regions. These strains grouped into one cluster with a genetic diversity of 1.9% between strains and a distance of 3.7-7% from other *P. carotovorum* clusters.

Clusters PcI and PcII, with a genetic distance of 3.7% to each other, are the most closely related *P. carotovorum* groups in both the MLSA and AFLP analyses. In the AFLP analyses, different banding profiles were observed for PcI and PcII. Both clusters were considered to be part of one clade by Ma *et al*. (2007). These two clusters are suggested to be formed by a variety of genetically different strains of the common *P. carotovorum* subsp. *carotovorum*. For better classification and deeper characterization of the strains of PcI and PcII, extensive analysis for their virulence variability is necessary.

The four *P.* carotovorum subsp. *odoriferum* (Gallois *et al*., 1992) strains group into PcIII and show low genetic variation (1%) between strains. The distances to the four other Pc clusters was significantly higher, with a range of 4.5-7.3%. These strains form an obvious subspecies based on their genomic differentiation. In a previous study (Ma *et al*., 2007) only one *P. carotovorum* subsp. *odoriferum* strain (SCRI482) was included. The strain clustered with other *P. carotovorum* subsp. *carotovorum* inside their clade II, so was not regarded as a different subspecies. Nevertheless, when combined with other three strains from this study, the strain grouped into a separate *P.* carotovorum subsp. *odoriferum* cluster. These results strongly confirm the necessity of using more than one strain representing genetically different taxa, especially subspecies, in a phylogenetic analysis.

The cluster PcIV or '*P. carotovorum* subsp. *brasiliensis*' (Duarte *et al*., 2004) is comprised of 8 strains from Brazil, America and Israel (Ma *et al*., 2007) as well as 10 strains from Peru, Syria, Germany and Japan. Recently, strains isolated in South Africa were identified as '*P. carotovorum* subsp. *brasiliensis*' (Merwe *et al*., 2010). The '*P.carotovorum* subsp. *brasiliensis*' strains were mainly isolated from potatoes in addition to other hosts, such as peppers, carrots and *Ornithogalum*. The genetic diversity within PcIV was estimated as 3.4%, and PcIV showed a distance of 4.8-7.1% to the other *P. carotovorum* clusters.

Two atypical *P. carotovorum* strains from Syria, C331 and C364.2, group into the clusters of either '*P. carotovorum* subsp. *brasiliensis*' or *P. carotovorum* subsp. *odoriferum* by AFLP analysis and MLSA phylogeny, respectively. These two strains, isolated from potatoes, are able to utilize a-methyl glycoside and reduce sucrose. Both characteristics are considered stable criteria for all strains of *P. carotovorum* subsp. *odoriferum* (Gallois *et al*., 1992) and for some strains of '*P. carotovorum* subsp. *brasiliensis*', especially strains identified by Duarte *et al*. (2004). These strains may indicate a new recombination of soft-rot isolates (Naylor *et al*., 2002).

Cluster PcV, including six strains, is comprised of strains from the monocotyledonous plants, *Saccharum arundinaceum*, *Zantedeschia aethiopica*, *Ornithogalum dubium* and *Ornithogalum dubium,* delineated into one monophyletic cluster along with strains from the dicotyledonous plants *Solanum tuberosum* and *Persea americana.* The monocot strains were grouped with two dicot strains at a genetic diversity

of 2.6%, and a distance of 6.8-7.3% to the other *P. carotovorum* clusters. These distantly grouped strains indicate a distinct cluster previously unreported. This cluster presents a new phylogenetic species including the strain *P. carotovorum* 106 (Ma *et al*., 2007) which was identified as an orphan taxon. A *P. carotovorum* taxon comprised strains isolated from monocot plants was also suggested by Yishay *et al*., (2008) using data from virulence tests, ITS-PCR banding patterns and 16S rRNA sequence analysis.

Strains belonging to the genus *Dickeya* demonstrated a clearly distinct group, by both AFLP and MLSA analyses, with an average genetic distance of 18.0% to all *Pectobacterium* strain clusters. These results were calculated from an analysis of 104 strains. The difference in the genetic diversity of 10.8% within the genus *Dickeya* (18 strains) when compared to the diversity within *Pectobacterium* (86 strains, 6% overall genetic diversity) may stem from sampling bias due to the high number of *Pectobacterium* strains included in the dataset, including several highly similar strains, thus reducing the average diversity when compared to the 18 *Dickeya* strains. Moreover, *Dickeya* was formerly divided into six species by Samson *et al*. (2005) and a new genetic clade, tentatively called *Dickeya solani* (Slawiak *et al*., 2009). This division supports our results that indicate the genus *Dickeya* being the most diverse among the soft-rot pathogens. We detected an unexpected diversity of 10.3% between strains of the species *D. dadantii*. As these strains were retrieved from GenBank under this name, it is possible that some of these strains have been misidentified. Their origin needs to be confirmed to exclude such a possibility.

The new strains isolated from Syria and investigated in this study are belonging to five phylogenetic groups namly; *P. c.* subsp. *carotovorum* (clusters PcI and PcII), '*P. c.* subsp. *brasiliensis*', (PcIV), *P. atrosepticum*, and *Dickeya* spp. The pathogenicity experiment results confirmed that none of the representative *Pectobacterium* clusters can cause soil disease but can induce severe stem maceration. The *Dickeya* spp. strains are the only soil pathogens and cause stem tissue maceration as well. Avoiding the source of the bacterial inoculation using sanitary procedures like pathogen-free irrigation water and agriculture machineries is considered the main control procedure toward stem infection of wounded plants. For soil pathogens and contamination which comes from the seed lots, there is a continuous need to select for more tolerant or resistant cultivars against locally isolated strains in each country (Perombelon, 2002; Czajkowski *et al*., 2009).

3. Taxonomic relatedness among *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum* and *P. carotovorum* subsp. *brasiliense* subsp. nov. [2]

3.1. Abstract

Pectobacterium carotovorum is a heterogeneous species of pectolytic, Gram negative bacteria that cause soft rot diseases of many agricultural crops and ornamental plants. In addition to the two named subspecies, *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *odoriferum*, a third subspecies, *P. carotovorum* subsp. *brasiliense*, was previously proposed. DNA-DNA hybridization confirmed that *P.c.* subsp. *brasiliense* is a different species from *P. wasabiae*, *P. betavasculorum,* and *P. atrosepticum,* with 28 %, 35 %, and 55 % similarity values, respectively, but is a member of the *P. carotovorum* species with 73-77 % similarity values. Sequencing the entire 16S rRNA of two polymorphic copies from strains of each of the *P. carotovorum* subspecies revealed sequence divergences among the three subspecies estimated at 1.5 % mean distance between *P. carotovorum* subsp. *odoriferum* and *P. carotovorum* subsp. *brasiliense*, 1.2 % between *P. carotovorum* subsp. *odoriferum* and *P. carotovorum* subsp. *carotovorum* and 0.9 % between *P. carotovorum* subsp. *brasiliense* and *P. carotovorum* subsp. *carotovorum*. The average 16S sequence diversity between *P. carotovorum* subsp. *brasiliense* and *P. carotovorum* subsp. *carotovorum* was lower than the maximum genetic distances between two sequence types obtained from the same strain, and lower than the maximum differences between sequence types within each subspecies separately. The 16S rRNA could not be used to discriminate among the subspecies, although it provided information on their relationships. Multi-locus sequence analysis based on eight house-keeping genes differentiated the subspecies and delineated two *P. carotovorum* subsp. *brasiliense* clades.

Clade I was comprised of strains isolated from Brazil and Peru while clade II included strains from Asia, North America and Europe. Strains in clade I but not clade II were phenotypically consistent with the original description of *P. carotovorum* subsp. *brasiliense* in that they produced reducing substances from sucrose and acid from α-methyl glucoside. The type *P. carotovorum* subsp. *brasiliense* 212 (= LMG21371 ^{T} = IBSBF1692 ^{T} = CFBP6617^{T}) was previously designated.

[2] Accepted for publication in the International Journal of Systematic and Evolutionary Microbiology (2011).

3.2. Introduction

Pectobacterium carotovorum, in the family *Enterobacteriaceae*, is a highly diverse species consisting of at least two valid names, *P. carotovorum* subsp. *carotovorum* (Gardan *et al*., 2003) and *P. carotovorum* subsp. *odoriferum* (Gallois *et al*., 1992), and a suggested third taxon, *P. carotovorum* subsp. *brasiliense,* (Duarte *et al*., 2004). Despite the lack of valid publication, the *P. carotovorum* subsp. *brasiliense* name has been used in more than ten publications since first published in 2004 (Duarte *et al*., 2004; Ma *et al*., 2007; Glasner *et al*., 2008; Naum *et al*., 2008; Czajkowski et al., 2009; Kim et al., 2009, van der Merwe et al., 2010; Williamson *et al*., 2010; Marquez-Villavicencio *et al*., 2011; Chapter II). Assigning strains to this taxon was based mainly on genetic information of the 16S-23S intergenic spacer region of the rRNA operon (IGS), 16S rRNA and MLSA of house-keeping genes.

P. carotovorum subsp. *brasiliense* was first described as causing blackleg disease on potatoes (*Solanum tuberosum* L.) in Brazil (Duarte *et al*., 2004) and has since been described as also causing soft rot in *Capsicum* annum L., *Onithogalum* spp., and *Daucus carota* subsp. *sativus as in* Chapter II. Strains of this taxon were isolated in USA, Canada, South Africa, Peru, Germany, Japan, Israel, and Syria. About 20 % of the *P. carotovorum* strains collected in Syria were identified as *P. carotovorum* subsp. *brasiliense* (Chapter II).

Glasner *et al*. (2008) showed that *P. carotovorum* subsp. *brasiliense* (CFBP6617^T) has a conserved core genome of 3.9 Mb in common with *P. carotovorum* subsp. *carotovorum* (WPP14) and *P. atrosepticum* (strain SCRI 1043). However, 13 % of genes in the chromosome of strain CFBP6617^T were found in neither *P. atrosepticum* nor *P. carotovorum* subsp. *carotovorum* (Glasner *et al*., 2008). They also suggested that *P. carotovorum* subsp. *brasiliense* should have species status because of its unique genomic organization, an observation which was confirmed by the AFLP analyses of Chapter II.

The objective of this study was to determine the validity of the suggested taxon *P. carotovorum* subsp. *brasiliense* using several different types of analyses. We considered both the classical approach to taxonomy using 16S rRNA sequence data as well as DNA-DNA hybridization (DDH) data to authoritatively propose and validate the *P. carotovorum* subsp. *brasiliense* nomenclature (Lapage *et al*., 1992).

3.3. Materials and Methods

Strain collection

Strains used in this study (Table 1) represented the three subspecies of *P. carotovorum, P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *brasiliense* and *P. carotovorum* subsp. *odoriferum*. *P. carotovorum* subsp. *carotovorum* strains were represented by isolates in two clusters from the previously reported MLSA study (Chapter II) and the type strain CFBP 2046^T. *P. carotovorum* subsp. *brasiliense* and *P. carotovorum* subsp. *odoriferum* strains from several geographical origins were obtained from various sources and also included the type strain of each taxon, *P. carotovorum* subsp. *brasiliense* 212 and CFBP 1878^T respectively.

MLSA Analyses

Sequence data for 22 *P. carotovorum* strains (Table 1) were used from the previously obtained sequence files (Chapter II). For an additional eight *P. carotovorum* subsp. *brasiliense* strains and the type strains CFBP 2046^T and CFBP 1878^T, fragments of eight conserved housekeeping genes (*mtlD*, *acnA*, *icd*, *mdh*, *pgi*, *gapA*, *proA* and *ropS*) were amplified using PCR as described previously (Chapter II). The fragments were purified from agarose gels and sent for sequencing without cloning. Sequencing was done by SeqLab (Göttingen-Germany). Sequences were submitted to GenBank under accession numbers JF926762 to JF926841.

The housekeeping gene sequences were tested for accuracy by Chromas software and aligned using CLUSTAL_W in Mega version 5.5 (Tamura, Peterson, Stecher, Nei, and Kumar 2011). Using all nucleotide sites of each partial gene sequence [*mtlD* (461 - 468 bp), *acnA* (347 bp), *icd* (553 bp), *mdh* (505 - 516 bp), *pgi* (543 bp), *gapA* (489 bp), *proA* (705 bp) and *ropS* (849 bp)], the lowest Bayesian information criterion was considered to describe the best substitution pattern and the General Time Reversible model (GTR) was chosen. Non-uniformity in evolutionary rates among sites modeled by using a discrete Gamma distribution (+G) and by assuming that a certain fraction of sites are evolutionarily invariable (+I) (Tamura *et al*., 2007; Nei & Kumar. 2000). Based on GTR+G+I an informative maximum likelihood tree was constructed in Mega 5.5 (Tamura *et al.,* 2007) with a bootstrapping test of 1000 replications (Felsenstein, 1985). Individual trees were not computed separately for each gene sequence of the 32 strains. (phylogenetic tree based on concatenated aminacid sequences is provided Figure S12)

16S rRNA sequence analyses

Twenty-five strains were subjected to 16S rRNA analysis: three *P. carotovorum* subsp. *odoriferum* strains including CFBP 1878^{T}, eight strains of *P. carotovorum* subsp. *brasiliense* and 14 strains of *P. carotovorum* subsp. *carotovorum* including CFBP 2046^{T}. The available genome sequence of strain *P. carotovorum* Pc1 (NCBI GenBank CP001657) was used to obtain the seven copies of the 16S rRNA gene sequences and a flanking region of about 200 bp. The sequences were aligned and used to design new primers for sequencing the entire gene. Primer pair Lpf/Rpr was selected from the flanking regions of the aligned consensus sequence, provided online as table S4. PCR was performed using a 30 µl reaction mix containing 2 µg µl^{-1} of each primer, 15 µl master mix (Phusion Flash F-548s, Biozym, Germany), 12 µl H_2O, and 3.5 µl DNA template (50 µg µl^{-1}). The PCR amplification program consisted of an initial denaturation at 98 ^{o}C for 10 s, 30 cycles of 98 ^{o}C for 5 s, 62 ^{o}C for 5 s and 72 ^{o}C for 50 s, and a final 10-min elongation step at 72 ^{o}C in a Biometra T-gradient thermocycler. Amplified fragments were detected by electrophoresis on a 1.5 % agarose gel and bands of about 1700 bp were purified from the gel using a gel extraction kit (SeqLab). The extracted DNA was cloned using CloneJET^{TM} PCR Cloning kit in the vector pGET1.2/blunt. Taking into account the presence of multiple polymorphic copies of the 16S rRNA operon in species of *Enterobacteriacae* and knowing that *P. carotovorum* encodes seven rRNA operons (Glasner *et al*., 2008), we sequenced up to four transformed clones for each of the 25 bacterial strains in order to represent more than one 16S rRNA sequence type per strain. The clones were grown at 37 ^{o}C for 4-5 h and the transformed plasmids were extracted using a standard plasmid extraction lab protocol as per the supplementary file in IJSEM Online (Appendix: Lab Protocol). The extracted plasmids were used for sequencing the inserted fragments using different primers, provided online (Table S4). Sequencing was performed by Seqlab using the extended Hot-Shot method [BigDye Terminator ready reaction mix v3.1 (ABI)]. Sequences were submitted to GenBank under accession numbers JF926716 to JF926761.

The appropriate sequenced segments of the transformed plasmids obtained using primers indicated in table (S4) were assembled by matching overlapping regions in Vector NTI software, and the chromatograms were checked for accuracy of the sequences using the same program. The 16S rRNA sequences representing all strains were analyzed by PubMLST (http://pubmlst.org/analysis/) to check for identical sequences. The deduced DNA sequences were aligned in Mega 5 (Tamura *et al*., 2007) and the phylogenetic

relationships were inferred by using the Maximum Likelihood (ML) method based on the Hasegawa-Kishino-Yano model (Hasegawa *et al*., 1985). A ML tree was constructed in Mega 5 representing all sequence types available (Figure S10). Additionally, a ML tree representing each of the 25 strains by one 16S rRNA sequence type was also constructed in Mega 5 using the Jukes-Cantor model (Jukes & Cantor 1969) (Figure S11).

DNA-DNA hybridization

To obtain genomic DNA, bacterial cells were disrupted using a French pressure cell (Thermo Spectronic). The DNA was purified from the crude lysate by chromatography on hydroxyapatite as described by Cashion *et al*. (1977). DDH was carried out as described by De Ley *et al*. (1970), with the modifications of Huss *et al*. (1983), using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostat 6x6 multicell changer and temperature control with an *in-situ* temperature probe (Varian). The experiments were conducted at the facilities of the German Collection of Microorganisms and Cell Culture (DSMZ).

Phenotypic assays

Carbon source utilization studies were carried out using the Microlog system (Biolog Inc., Hayward). Twenty-one strains were assayed for oxidation of the 95 carbon sources in the GN2 microplates as suggested by the manufacturer. Microplates were inoculated with 150 µl of suspension per well at 27 ^{o}C. Additionally, all strains were assayed by tests commonly used for differentiating *Pectobacterium* subsp.: acid production from maltose and α-methyl-D-glucoside, reducing substances from sucrose, growth at 37 ^{o}C, erythromycin sensitivity, tolerance to 5 % NaCl, lactose fermentation and gas production from D-glucose (Schaad, 1988).

3.4. Results

MLSA Analyses

The MLSA analysis revealed specific clusters of strains (Figure 1) with the *P. carotovorum* subsp. *carotovorum* strains grouping into the two clusters (Clades III, IV) identified in the previous MLSA study (Chapter II). The strain CFBP 2046^{T} fell in the infrasubspecies clade IV with a high level of similarity to strain SCRI 2 (NCPPB275) which is still misidentified as *P. atrosepticum* in different international collections (Chapter II). The *P. carotovorum* subsp. *brasiliense* strains also grouped into two infrasubspecies clades, I and II. Strains in clade I were from Brazil and Peru while clade II

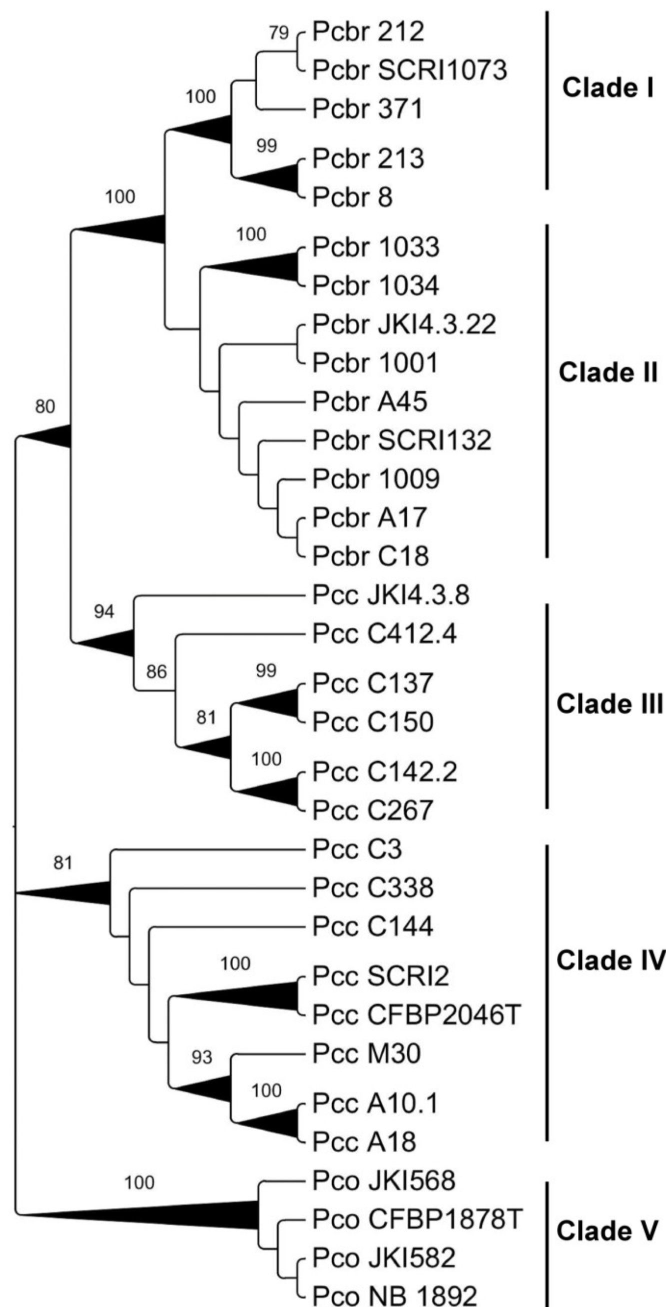
was comprised of strains from Europe, Asia, and North America, the latter having been identified previously as *P. carotovorum.* subsp. *brasiliense* by MLSA. *P. carotovorum* subsp. *brasiliense* strains 1033 and 1034 from Canada were more dissimilar from the type strain *P. carotovorum* subsp. *brasiliense* 212 than the other Canadian strains. All the other strains from Canada (including strain 1001 that had a low DDH similarity value) were clearly assigned to clade II. Strains of *P. carotovorum* subsp. *odoriferum*, assigned to clade V, formed a separate homogeneous cluster.

16S rRNA Analyses

The 16S rRNA analyses were based on exactly 1530 bp representing the complete sequence of the gene. Interesting motifs found in the flanking regions had no correlation to the sequences of the entire 16S rRNA and were not considered. The 16S rRNA sequences were confirmed either by sequencing the same sequence type two times or by sequencing the reverse strand. The informative parsimony nucleotide sites were limited to 35 sites distributed mainly in four regions of the gene (positions 65 – 91, 210, 445 – 489 and 1001 – 1039, *Escherichia coli* 16S rRNA gene sequence numbering is indicated) provided online (Table S5), while there were 12 sites at which base substitution only occurred once. The single base substitutions were not included in the phylogeny computations. The overall diversity among the 35 sequence types obtained from the 25 strains of the three subspecies was 0.825 %, indicating the strains all belong to the same species. A maximum difference of 1.871 % was observed between the 16S rRNA sequence of *P. carotovorum* subsp. *brasiliense* 371 (a Brazilian isolate) and the sequence of the strain CFBP 1878^T as well as strain JKI 582 (a Swiss isolate). Only two 16S rRNA sequence types were obtained for the three *P. carotovorum* subsp. *odoriferum* strains, and they differed in only one nucleotide (at position 77), indicating 0.065 % diversity. Whilst maximum diversity in 16S rRNA sequences within subspecies were 1.39 % and 1.125 % for *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliense*, respectively, maximum diversity in the different copies of the gene within the same strain were 0.727 % and 1.057 % for *P. carotovorum* subsp. *carotovorum* (strain C3) and *P. carotovorum* subsp. *brasiliense* (strain 371), respectively. For example, the two sequences obtained from *P. carotovorum* subsp. *brasiliense* C18 differed in 10 nucleotides, 8 at one region and 2 at two different regions within the gene. In the phylogenetic analysis of the 16S rRNA sequence *P. carotovorum* subsp. *odoriferum* strains clustered separately from *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliense* but the latter two subspecies were not clearly differentiated from one another (Figure S10 and Figure S11).

Figure 1.

Dendrogram showing evolutionary relationships among *P. carotovorum* subspecies based on multi-locus sequence analysis of eight housekeeping gene fragments applied to 32 strains by the Maximum Likelihood method based on the (GTR+G+I) specific model. The bootstrap consensus tree is inferred from 1000 replicates. There were a total of 4354 positions in the final dataset.



Bootstrapping values less than 70 % were cut off.

DNA-DNA hybridization

Strain *P. carotovorum* subsp. *brasiliense* 212, was used as the template for DDH with *P. carotovorum* CFBP 2046^{T}, *P. wasabiae* CFBP 3304^{T}, *P. atrosepticum* CFBP 1526^{T}, and *P. betavasculorum* CFBP 2122^{T}, as well as for DDH analysis of an additional five *P. carotovorum* subsp. *brasiliense* strains. The results confirmed that *P. carotovorum* subsp. *brasiliense* was a different species from *P. wasabiae*, *P. atrosepticum* and *P. betavasculorum* with homologies of < 70 %, but with a high level of similarity (> 70 %) to *P. carotovorum* subsp. *carotovorum* suggesting alignment with this species (Table 2). The five *P. carotovorum* subsp. *brasiliense* strains CFBP6617^{T}, A17, 8, 317 and 132 were confirmed as belonging to the same taxon using the threshold value of 70 % DNA-DNA similarity for defining bacterial species as per the recommendation of the *ad hoc* committee of bacterial systematics (Wayne *et al*., 1987) (Table 2). The identity of strain *P. carotovorum* subsp. *brasiliense* 1001 with low DDH values is uncertain and needs to be confirmed using a different kind of analysis.

Phenotypic assays

Twenty-one carbon sources were utilized by all strains in all subspecies, and an additional 8 carbon sources were used by all strains except strain *P. carotovorum* subsp. *brasiliense* 132 isolated in Japan from carrot. Strains in both *P. carotovorum* subsp. *carotovorum* clades (clade III and IV) differed dramatically in their ability to use different carbon sources ranging from about 30 to 63 compounds for strains within each of the two clades. However, the carbohydrate utilization patterns did not differentiate between *P. carotovorum* subsp. *carotovorum* strains in the two clades with one exception that strains in clade III utilized glucose-1-phosphate along with a limited number of strains in all other clusters including *P. carotovorum* subsp. *carotovorum* clade IV.

Strain *P. carotovorum* subsp. *brasiliense* SCRI1073 (clade I) utilized 75 carbon sources and its phenotype was consistent with the original description of the subspecies based on Brazilian strains in that it produced reducing substances from sucrose and acid from maltose and α-methyl glycoside (Table 3). However, its pattern of carbohydrate utilization differed from that of the Brazilian strains as reported by Duarte *et al.* (2004). *P. carotovorum* subsp. *brasiliense* strains in clade II differed from clade I strains in that they did not produce reducing substances from sucrose or utilized α-methyl glycoside. Furthermore, unlike clade I strains, clade II strains also neither produced acid from maltose nor utilized acetic and lactic acids.

The three *P. carotovorum* subsp. *odoriferum* strains utilized 39, 40 and 42 carbon sources. All *P. carotovorum* subsp. *odoriferum* strains utilized tween 80, D-psicose, and D-sorbitol, while only a limited number of strains in all other clades utilized these compounds.

3.5. Discussion

Establishment of *P. carotovorum* subsp. *brasiliense* as a separate taxon, initially based on differences in 16S rRNA sequence, amplification of the intergenic spacer region in PCR, and analysis of biochemical reactions (Duarte *et al*., 2004), was validated by the MLSA analysis of Ma *et al*. (2007). The study of Ma *et al.* (2007), moreover, extended the diversity within the taxon by including strains from North America. A follow-up study by Glasner *et al.* (2008) using a pan-genomic approach suggested that *P. carotovorum* subsp. *brasiliense* is phylogenetically distinct from the other pectobacteria and referred to it as a separate species. The notion of a separate species status for *P. carotovorum* subsp. *brasiliense*, however, was not sustained by our DDH analysis which confirmed the subspecies status of the taxon as initially described by Duarte *et al*. (2004). It is well known that DDH has limited capacity for differentiating among strains belonging to the same species and was, therefore, not expected to differentiate among the subspecies (Vandamme *et al*., 1996).

Our analysis of 16S rRNA sequences of *P. carotovorum* subsp. *brasiliense* strains from both Brazilian and non-Brazilian sources failed to consistently substantiate their differentiation from other *P. carotovorum* strains on this basis (Figure S11). In the study of Gardan *et al*. (2003) on *Pectobacterium* species, the 16S rRNA similarity values were not analyzed and now it is known that analysis of partial 16S rRNA sequences fail to differentiate strains of *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliense* (Naum *et al*., 2008). With seven copies of the ribosomal gene in the chromosome, it is unknown how many different homologs of the 16S rRNA gene are present in a single strain. Both *P.c.* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliense* displayed 20 and 13 different 16S rRNA sequence types, respectively, in the 14 and 8 strains that we studied. None of 16S rRNA sequence types obtained from strains belonging to different subspecies was identical to one another, but two sequence types obtained from the same strain even placed distantly from one another in the phylogenetic tree (Figure S10). This observation indicates that the apparent phylogenic relationships will

differ depending on which 16S rRNA sequence type of each strain is used in the analysis, and brings into question the accuracy of resulting evolutionary trees. The relative diversity in the 16S rRNA sequence types obtained from *P. carotovorum* subsp. *brasiliense* and *P. carotovorum* subsp. *carotovorum* strains and the low genetic distances prevented clustering of strains into individual groups representing the subspecies (Figure S11).

Informative nucleotides among the 16S rRNA sequence types, although few in number, are generally at the same positions for each of the three subspecies (Table S5). With the possibility of any of the four bases being present, there is a high probability of finding the same base in the same position for each of the sequence types representing strains of different subspecies. Base substitutions at discriminating nucleotide sites are exemplified at position 445 where all *P. carotovorum* subsp. *carotovorum* sequence types have A but all *P. carotovorum* subsp. *brasiliense* sequence types have G, and at position 489 where all *P. carotovorum* subsp. *carotovorum* sequence types have T but all *P.c.* subsp. *brasiliense* sequence types have C. However, taxon-related base substitutions such as those at positions 445 and 489 are not sufficient within the 16S rRNA gene to separate the two subspecies within the phylogenetic tree (Figure S11). Such consistent base substitutions, however, can explain why strains of both *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliense* occur within the same cluster and as in one case *P. carotovorum* subsp. *brasiliense* sequence types clustered clearly within a predominantly *P. carotovorum* subsp. *carotovorum* 16S group (Figure S10).

A threshold of 97 % binding is generally accepted as the cut-off value required for discriminating species. However, the low level of 16S rRNA sequence diversity we observed among the three *P. carotovorum* subspecies was in agreement with what was previously reported among taxa of the family *Enterobacteriaceae* and different studies reported very low discriminatory values which can not guarantee species identity (Hauben *et al*., 1998; Kwon *et al*., 1997; Spröer *et al*., 1999; Ibrahim *et al*., 1993; Tindall *et al*., 2010). Staley (2006) confirmed that 16S rRNA lacks resolution below the genus level and that MLSA better resolves bacterial speciation. We did observe high sequence stability among the three strains of *P. carotovorum* subsp. *odoriferum* used in our study and, as expected, this translated into distinct phylogenetic clusters for the subspecies in both the 16S rRNA and MLSA dendrograms (Figure 1 and Figure S11). The uniformity among *P.*

carotovorum subsp. *odoriferum* strains is consistent with its narrow host range on celery, chicory and hyacinth and restricted distribution in Europe (Gallois *et al*., 1992).

On the basis of our MLSA results, *P. carotovorum* subsp. *brasiliense* was differentiated as a separate phylogenetic group from *P. carotovorum* subsp. *carotovorum* in contrast to the DNA hybridization data and the 16S rRNA phylogeny. Of the 4354 nucleotides analysed per strain by MLSA, 465 were polymorphic informative sites and were used to define the three subspecies, *P. carotovorum* subsp. *odoriferum*, *P. carotovorum* subsp. *carotovorum*, and *P. carotovorum* subsp. *brasiliense*. The 15 % polymorphism in the MLSA sequences was significantly higher than the 3 % polymorphism in the 16S rRNA sequences reflecting a higher rate of divergence among the metabolic genes compared to the ribosomal operon and probably represents adaptation of various strains to specific environmental niches (Achtman, 2008). The better resolution obtained by MLSA based on different regions within the genome, interestingly revealed two clades, designated *P. carotovorum* subsp. *brasiliense* I and *P. carotovorum* subsp. *brasiliense* II, within the subspecies but belonging to the same monophyletic group based on DDH. In other words, MLSA distinguished among strains with different adaptations at the infrasubspecies level.

Seven of the housekeeping genes analyzed in this study are protein-encoding. This means it is likely that MLSA measures a degree of phenotypic relatedness that cannot be resolved by analysis of 16S rRNA. It is already known that a few 16S rRNA groups are without phenotypic justification and this is another example of a disconnection between phylogeny based on 16S rRNA sequences and phenotypic relationships (Woese, 1987). 16S rRNA phylogeny provides a means of defining natural groupings of bacteria that is purely genotypic and independent of any phenotypic definition. Technically, sequencing of a sufficient number of housekeeping genes (protein-encoding sequences) shared by taxa under investigation can provide a higher level of resolution than the DNA-encoding genes.

Phenotypic discrimination among the three subspecies using the traditional microbiological methods is challenged by the diversity of strains within each subspecies. Strains of *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliense* varied in their ability to utilize a number of carbon sources. Some strains of each subspecies utilized a high number of carbohydrates while others utilized only a few. Perhaps the nucleotide polymorphisms affect gene regulation and subsequent metabolic

processes, reflecting adaptation of specific strains to different environmental or ecological niches (Glasner *et al*., 2008).

Clade I of *P. carotovorum* subsp. *brasiliense* in the MSLA dendrogram includes all the strains of the subspecies isolated from Brazil and Peru. Phenotypically, these strains conform to the discriminating criteria of Duarte *et al*. (2004) and so far have only been isolated from potato (El Tassa and Duarte, 2004). In contrast strains in clade II are world-wide in distribution and were isolated from various hosts including *Solanum tuberosum* (Ex. Canadian and Syrian strains), L., *Daucus carota* subsp. *sativus* (SCRI 132), *Capsicum anuum* L. and *Onithogalum* spp. (Chapter II). Phenotypically, the clade II strains differ from the discriminating criteria of Duarte *et al*. (2004), particularly their inability to produce reducing substances from sucrose or acid from α-methyl glucoside, characteristics that had initially caused the clade I strains to be considered 'high temperature' variants of *P. atrosepticum*. Van der Merwe *et al*. (2010) reported the potato blackleg disease as being caused by *P. carotovorum* subsp. *brasiliense* in South Africa but data presented in the paper was insufficient to determine the clade affiliation of these strains.

In summary our DDH results clearly show that *P. carotovorum* subsp. *brasiliense* strains are taxonomically aligned with the *P. carotovorum* species but at varying dis-reassociation values (Table 2). The MLSA resolved two clades within *P. carotovorum* subsp. *brasiliense* as it did for *P. carotovorum* subsp. *carotovorum* (Figure 1) but whether each clade ought to be considered a separate subspecies requires further study. Differentiation of *P. carotovorum* subsp. *brasiliense* as a separate subspecies provides some clarity to the heterogeneity within the *P. carotovorum* species (De Boer, 2003).

Description of *Pectobacterium carotovorum* subsp. *brasiliense* subsp. nov.

Pectobacterium carotovorum subsp. *brasiliense* bra.si.li.en'se. N.L. neut. adj. belonging to Brasilia is a subspecies of *P. carotovorum* and phenotypically conforms to the species description. It is Gram-negative and pectolytic, producing characteristic pits on pectate-based selective media, such as crystal violet pectate (CVP) medium. On CVP and nutrient agar colonies of *P. carotovorum* subsp. *brasiliense* are indistinguishable from other *Pectobacterium* species and subspecies. The initial description of the subspecies indicated that *P. carotovorum* subsp. *brasiliense* was distinguishable from *P. carotovorum* subsp. *cartovorum* by ability to produce reducing substances from sucrose and acid from

α-methyl-glucoside and maltose. It grows at 37 ^{o}C, distinguishing it from *P. atrosepticum*. Differentiation between the two *P. carotovorum* subspecies was further based on sequence of the 16S rRNA (Duarte *et al*., 2004) and confirmed by MLSA (Ma *et al*., 2007). Subsequently, strains were isolated that were identified as *P. carotovorum* subsp. *brasiliense* because the intergenic spacer region of the ribosomal operon was amplified in a *P. carotovorum* subsp. *brasiliense*-specific PCR assay and they grouped with *P. carotovorum* subsp. *brasiliense* in 16S rRNA and MLSA analyses but did not produce reducing substances from sucrose and acid form α-methyl glucoside. The results presented in this study show that MLSA differentiates between two clades of *P. carotovorum* subsp. *brasiliense*, placing the strains conforming to the original description in clade I and the remainder in clade II.

All strains ferment lactose, tolerate 5 % NaCl, grow at 37 ^{o}C, and utilize D-trehalose and D-melibiose and with few exceptions D-galacturonic acid and can not utilize glucose-1-phosphate, malonic acid, dextrin and D-arabitol. Strains in clade II cannot utilize acetic acid and D, L-lactic acid whereas it can utilize N-acetyl-D-glucosamine. In contrast strains in clade I can utilize succinamic acid and lactic acid but cannot utilize N-acetyl-D-glucosamine. The strain *P. carotovorum* subsp. *brasiliense* 212 (= LMG21371 ^{T} = IBSBF1692 ^{T} = CFBP6617^{T}) designated as the type strain of the subspecies.

4. *Pectobacterium aroideae* sp. nov. formerly known as *Erwinia aroideae* (1904), a soft rot pathogen of monocotyledonous plants

4.1. Abstract

The heterogeneity among strains classified as *Pectobacterium carotovorum* suggests that some of the strains ascribed to this species ought to be reclassified. We revived the specific epithet of the name *Erwinia aroideae*, first used to describe strains isolated from monocotyledonous plants, for pectobacteria isolated from soft rot lesions of monocots, that have distinct intergenic spacer regions and 16S rDNA sequences, and group separately in multilocus sequence analysis (MSLA) of conserved metabolic genes. DNA-DNA hybridization (DDH) values ranged from 30.4 to 64.1 % between these strains and type strains of other *Pectobacterium* spp. These strains also differed from *P. carotovorum* in their utilization of dextrin, glucuronamide, and α-ketoglutaric acid. Strains of pectobacteria causing soft rot of monocotyledonous plants and having distinct genomic and biochemical features, formerly classified as members of *P. carotovorum*, represent a novel species for which the name *Pectobacterium aroideae* sp. nov. is proposed; SCRI 109^T, (NCPPB 929^T, LMG 2417^T, ICMP 1522^T) is designated the type strain.

4.2. Introduction

Multi-locus sequence analysis (MLSA) of strains in the pectolytic enterobacterial genus, *Pectobacterium*, using eight housekeeping genes (*acnA*, *icd*, *pgi*, *gapA*, *mtlD*, *mdh*, *proA* and *rpoS*) confirmed the validity of the previously designated species and subspecies, i.e. *Pectobacterium atrosepticum*, *Pectobacterium betavasculorum*, *Pectobacterium wasabiae* and *Pectobacterium carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum* and *P. carotovorum* subsp. *brasiliense* (Ma *et al*,. 2007). The MLSA studies also revealed extensive genomic heterogeneity within the *P. carotovorum* species (Ma *et al*., 2007) (Chapter II). One distinct cluster of *P. carotovorum* strains, designated as cluster PcV, was identified in chapter II as consisting mostly of strains isolated from monocotyledonous plants. Interestingly, three of the six strains delineated in the PcV cluster were initially reported as *Erwinia aroideae*. The specific epithet, *E. aroideae,* was first used by Townsend in 1904 for soft rot bacteria from the monocotyledonous arum or calla lily (*Zantedeschia spp.*) (Dowson, 1957). Holdeman & Burkholder (1956) also

recognized *E. aroideae* isolates as constituting a subgroup within its species (i.e. *Erwinia carotovora var. aroideae*) but subsequently Graham (1964) and Dye (1969) considered *E. aroideae* to be synonymous with *E. carotovora* var. *carotovora*. Various studies generating serological (Eldor, 1941), and molecular data (Starr *et al*., 1969; Brenner 1973) confirmed that the soft rot pathogen, *E. aroideae*, as described in the 7th edition of Bergey's Manual should not be considered a separate species. The status of *E. aroideae* remained uncertain for about 70 years until it was agreed to expel it from the list of plant pathogenic bacteria and consider the name to be a synonym for *E. carotovora* (now classified as *P. carotovorum*) as in the 8th edition of Bergey's Manual. Currently *P. carotovorum* is a diverse species as exemplified by the 62–100 % DNA-DNA hybridization between the type strain of the species and 14 other strains within the species, two of which were originally designated as *E. aroideae* (Gardan *et al*., 2003). In this paper we propose to revive the specific epithet of *E. aroideae* for a new species *Pectobacterium aroideae* sp. nov. to accommodate the distinctive pectobacteria that mainly, but not exclusively, cause soft rot diseases of monocotyledonous plants.

4.3. Materials and Methods

As in chapter II, the partial sequences of eight housekeeping genes (*acnA, icd, gapA, mdh, mtlD, pgi, proA* and *rpoS*) were amplified, purified, and sequenced for each of the type strains of *P. atrosepticum*, *P .betavasculorum* and *P. wasabiae,* accession numbers JN600333 to JN600355. Additionally, the corresponding 56 sequences of the four putative *P. aroideae* strains (Table 1) and of the type strain of each of three subspecies of *P. carotovorum* were retrieved from the NCBI GenBank (Chapter II and III). The sequence data were aligned using CLUSTAL_W in Mega 5.5 (Tamura *et al*., 2007) and used to draw the topology of the phylogenetic tree using the best evolutionary model Tamura-Nei (Tamura & Nei, 1993).

The phylogenetic derived tree based on the aminoacid gene sequences from the concatenated protein coding genes (*acnA, icd, gapA, mdh, mtlD, pgi, proA* and *rpoS*) was computed (figure S12) after translation of each gene sequence in a separated alignment. The aminoacid data of all genes can also be retrieved from the UniProt data base.

The complete 16S rDNA gene for each strain was amplified using primer pair Lpf/Rpr, purified, cloned and sequenced (Chapter III). The phylogenetic tree of the natural

relationships of 12 *Pectobacterium* strain represented by 24 16S rDNA homologs was computed using Mega 5.5 version based on the Hasegawa-Kishino-Yano best model. (Figure 3).

PCR amplification and restriction digestion of the ITS

The Intergenic transcribed spacer (ITS) was amplified using the primers G1 and L1 from the 63 strains included in chapter II. Ten µl of the amplified product was digested with each of the restriction enzymes *Rsa*I and *Cfo*I (*Hha*) (Invitrogen) (Toth *et al*., 2001). Digested samples (10 µl) were electrophoresed through a 2% agarose gel in TAE buffer for 2 h at 100 V, and the fragment size estimated in comparison to a 50bp ladder (Fermentas).

Physiological assays

Forty two strains were tested for the ability to utilize pectin on CVP medium and subjected to the carbon source utilization study using the Microlog system (Biolog Inc., Hayward) of oxidation of the 95 carbon sources in the GN2 microplates as in chapter III. Additionally, all strains were assayed by tests commonly used for differentiating *Pectobacterium* species, such as acid production from maltose and α-methyl-D-glucoside, reducing substances from sucrose, growth at 37 ^{o}C and 29 ^{o}C, erythromycin sensitivity, tolerance to 5 % NaCl, lactose fermentation and gas production from D-glucose (Schaad, 1988).

Pathogenicity assay

The pathogenicity of the 63 strains (Chapter I) including strains in table 1 were tested against dicotylendonous and monocotyledonous host plants, potato (*Solanum tuberosum* L.), pepper (*Solanum capsicum* L.), onion (*Allium cepa* L.), pea (*Pisum sativum* L.) and radish (*Raphanus sativus* var. *longipinnatus*). The plant organs (tuber, fruit, bulb, pod fruit, and root, respectively) were disinfected with 1% NaOCl for 30 s each, and rinsed with sterile distilled water. The plant slices were inoculated in the middle using a Hamilton syringe with 5.0 µl 10^{8} CFU ml^{-1} ($O.D_{260}$ =0.24), 24 h old bacterial cells. Slices were put on a wet sterilized filter paper fit in the Petri dishes and incubated at 27 ºC. Tissue decays were measured and evaluated for symptoms up to 6 days. All virulence assays were repeated twice.

Table 1.

Putative *P. aroideae* strains used in this study

Strain designation	NCPPB	LMG	ICMP	Host	Origin	Isolation
SCRI 109	929	2417	1522	*Zantedeschia aethiopica*	South Africa	1959
SCRI 121	1640	2426	1606	*Saccharum* spp.	Jamaica	1963
SCRI 3	435	2383	1607	*Solanum tuberosum*	Zimbabwe	1955
SCRI 102	547	2414	1520	*Persea americana*	Israel	1955

4.4. Results and discussion

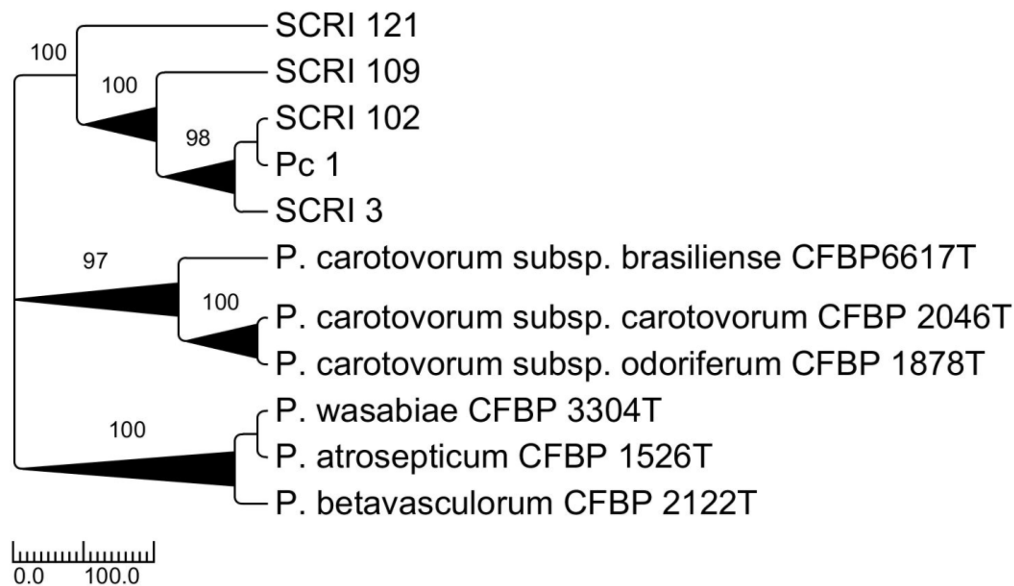
Four strains, putatively belonging to the new species, *P. aroideae*, were included in this study (Table 1). Based on data generated from different collections (NCPPB, LMG, ICMP) strains SCRI 102, SCRI 109, and SCRI 121 (Chapter II) were first designated as *Erwinia aroideae* and later as *P. carotovorum,* while strain SCRI 3 was first identified as *P. atrosepticum* and later as *P. carotovorum* (Chapter II) because its DNA was not amplified by PCR using primers specific for *P. atrosepticum* (De Boer & Ward, 1995), and genetically it clearly belongs to the other putative *P. aroideae* strains in AFLP analyses (Chapter II). For comparison, we included in this study the type strain of each of the three species of the genus *Pectobacterium*: *P. atrosepticum* (CFBP 1526^{T})*, P. betavascularum* (CFBP 2122^{T})*,* and *P. wasabiae* (CFBP 3304^{T}). Data for *P. carotovorum* were represented by the type strains of the three subspecies, namely *P. carotovorum* subsp. *carotovorum* (CFBP 2046^{T}), *P. carotovorum* subsp. *odoriferum* (CFBP 1878^{T}), and *P. carotovorum* subsp. *brasiliense* (CFBP6617^{T}) as in Chapter III.

Analysis of the concatenated eight gene sequences revealed a mean evolutionary diversity for the entire population of *Pectobacterium* represented by the five type strains including strain SCRI 109 of 6.5 %. The MLSA sequence data served to estimate the similarity between the suggested species *P. aroideae* and each of the three *Pectobacterium* species, *P. atrosepticum*, *P. betavasculorum* and *P. wasabiae* at 95 %, 95 %, and 96 % respectively, and with the three *P. carotovorum* subspecies, *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum* and *P. carotovorum* subsp. *brasiliense* at 95 %, 95 %, and 94 %, respectively, demonstrating a considerable distance in the metabolic lineage in comparison to the value of 97 % cut-off used for considering a new species based on the ribosomal operon sequence (Tailliez *et al*., 2010). Out of 68 *Pectobacterium* strains belonging to all described species and subspecies in the

genus, the *P. aroideae* strains also clustered in its own group in trees computed individually for each of the eight housekeeping genes (Chapter II). The aminacid evolutionary tree preserved the species status of the *Pectobacterium aroideae*. The aminacid evolutionary is based on lower number of informative sites because an exchange of the 3rd. codon position in the nucleotide has often no effect on the phenotype (the aminacid in the protein). This is why it has more authority to suggest evolutionary events than nucleotide sequence data and is becoming more essential in taxonomic studies.

Figure 1.

Molecular phylogenetic analysis of MLSA data of eight house-keeping genes of the type strains of the four *Pectobacterium* species; *P. atrosepticum*, *P. betavascularum*, *P. wasabiae*, *P. carotovorum* and the subspecies *P. carotovorum* subsp. *brasiliense* and *P. carotovorum* subsp. *odoriferum* and five representative strains of the novel species P. aroideae. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. Bootstrap test of 1000 replicates are shown. Branches corresponding to partitions reproduced in less than 70 % bootstrap replicates are collapsed.

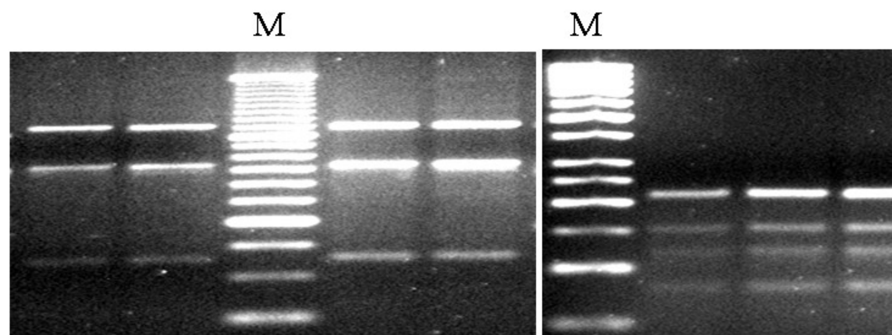


ITS-PCR restriction fragment length polymorphism (ITS-PCR-RFLP) analyses (Toth *et al*., 2001) has been used as a rapid and accurate method for differentiating among strains of soft rot *Pectobacterium*. We compared the RFLP patterns of the four putative *P.*

aroideae strains to patterns produced by the other pectobacterial species and subspecies (Chapter II). Three and four bands of various lengths (130, 360, 560 bp and 80, 120, 160, 210 bp) were resolved in digests obtained using *Rsa* I and *Hha* restriction enzymes, respectively. The banding profiles of the four *P. aroideae* strains were identical to one another (Figure 2 and Figure S12) and different from the banding profiles produced by the other species and subspecies of *Pectobacterium* and *Dickeya* spp.

Figure 2

In the left gel, ITS–PCR–RFLP amplification patterns of the putative *P. aroideae* strains SCRI 121, SCRI 109, SCRI 3, and SCRI 102, digested with *Rsa*I restriction enzyme and produced three variable band lengths of 130, 360, 560 bp, **in the right gel**, ITS–PCR–RFLP amplification patterns of putative *P. aroideae* strains SCRI 121, SCRI 109, and SCRI 3, digested with *Hha* restriction enzyme and produced variable band lengths of 80, 120, 160, 210 bp.

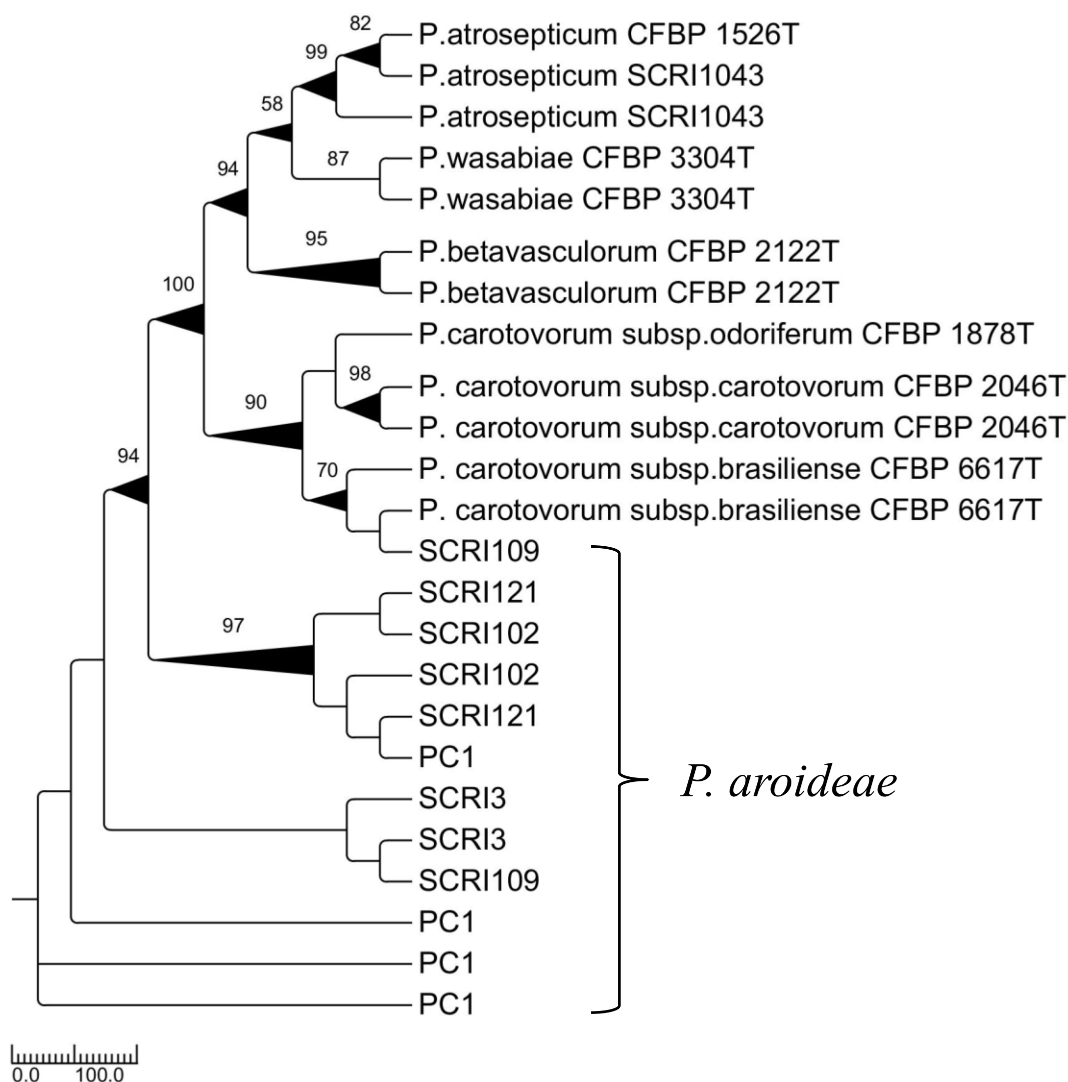


Two polymorphic 16S rDNA clones derived from each of the putative four *P. aroideae* strains and each of the type strains of *P. betavasculorum*, *P. wasabiae* and *P. atrosepticum* were sequenced in this study by the method reported previously (Chapter III) accession numbers JN600320 to JN600332. Five of the 16S rDNA gene occurring in the type strain of each *P. carotovorum* subspecies, four sequence types occurring in strain Pc1 [Pc1 designated as *P. carotovorum* (NCBI GenBank accession number CP001657) and re-designated in this study as *P. aroideae* strain, Pc1 isolated from *Ornithogalum dubium* in Israel]; and two sequence types found in strain *P. atrosepticum* SCRI 1043 (NCBI GenBank accession number FJ176937), were retrieved from the NCBI GenBank and aligned. The alignment of the 24 sequence types using Mega 5.5 and analyzed in CLUSTAL_W with the best fit model of Hasegawa-Kishino-Yano (Hasegawa *et al*., 1985) showed that seven sequence types of the 16S rDNA gene from the four putative *P.*

aroideae strains formed a distinct phylogenetic clade (Figure. 3). Pairwise comparison (Tamura, 2004) of the entire 16S rDNA sequence revealed similarity as low as 79.96 % and 79.99 % between the sequence types in strain SCRI 121 and *P. carotovorum* subsp. *odoriferum* and between sequence types in strain SCRI 102 and *P. betavasculorum*, respectively.

Figure 3.

Molecular phylogenetic analysis of entire 16S rDNA of 33 nucleotide sequence types (I, II). The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model. Bootstrap test of 1000 replicates are shown. Branches corresponding to partitions reproduced in less than 70 % bootstrap replicates are collapsed.



NCBI-Blast of the entire 16S rDNA sequence of strain SCRI 102 revealed a maximum similarity of 99 % with those of *P. carotovorum* strains isolated from monocotyledonous plant families (GenBank accession numbers HM622347.1, FJ593025.1, DQ886670.1, DQ785511.1, DQ785510.1, HM622345.1, FJ593031.1, HM622348.1, FJ906790.1, and HM622346.1). For example, strains SXR1 (DQ785511) and ZJR1 (DQ785510) were isolated from *Pinellia ternata* in China (Hu *et al*., 2007). Partial 16S rDNA sequence analysis confirmed the heterogeneity in the 16S rDNA operon allowing identification of the specific clade harbouring strains mostly but not exclusively isolated from soft rot lesions in monocotyledonous plants (Hu *et al*., 2007; Yishay *et al*., 2008; Wu *et al*., 2010; Zhu *et al*., 2010).

The wet DNA-DNA reassociation experiments were conducted by the DSMZ using strain SCRI 102 as the template for hybridization with each type strain of the four *Pectobacterium* species, *P. atrosepticum*, *P. betavasculorum*, *P. wasabiae* and *P. carotovorum* (Gardan *et al*., 2003). Further reassociation experiments were done within the four strains of *P. aroideae*. The results (Table 2 and 3) confirmed that the four strains assigned to the new species should be considered a separate species as per the phylogenetic definition of bacterial species of Wayne *et al*. (1987). High similarity values of 106.5 (103.8) % between strains SCRI 3 and SCRI 102 clearly was consistent with the identical AFLP banding profiles for the two strains (Chapter II).

Table 2.

Percent similarity of wet pairwise DNA-DNA hybridization of strain SCRI 102 against four type strains of the four species of *Pectobacterium* (Gardan *et al*., 2003) and DNA-DNA similarity determined by *in silico* estimation based on genome sequence of strain Pc 1 against genome sequences of *P. carotovorum* WPP 14, *P. carotovorum* subsp. *brasiliense* 212, *P. atrosepticum* SCRI 1034, and *Pantoea ananatis* LMG20103.

Type strains	Wet-DDH (DSMZ)	*In silico* DDH estimate (regression-based)
Pectobacterium wasabiae	**30.4 (36.4) %**	**X**
Pectobacterium betavasculorum	**43.6 (45.1) %**	**X**
Pectobacterium atrosepticum	**45.5 (46.5) %**	**42.54%**
Pectobacterium carotovorum	**64.1 (59.3) %**	**47.67%**
P. carotovorum* subsp. *brasiliense	**X**	**49.22%**
***Pantoea ananatis* LMG20103**	**X**	**11.39%**

Values in parentheses are results of measurements in duplicate

Table 3.
Percent similarity determined by DNA-DNA hybridization among strains of *P. aroideae.*

Strain designation	SCRI 109	SCRI 102	SCRI 3	SCRI 121
SCRI 109	X	X	X	X
SCRI 102	84.0 (79.6)	X	X	102.4 (100.8)
SCRI 121	80.8 (84.0)	102.4 (100.8)	81.0 (77.7)	X
SCRI 3	76.4 (70.6)	106.5 (103.8)	X	81.0 (77.7)

Based on alignment-free distance calculation and using the available whole genome sequence of the *P. aroidea* strain, Pc1 (NCBI GenBank CP001657), we used an *in silico* approach to infer genome-to-genome distances (GGD) (Auch, *et al*., 2010). Distances were calculated using NCBI-BLAST version 2.2.18 program. The query strain Pc1 was used as the template to calculate its GGD from each of *P. carotovorum* WPP14, *P. carotovorum* subsp. *brasiliense* CFBP 6617, *P. atrosepticum* SCRI 1043, and *Pantoea ananatis* LMG20103 as an out-group strain. The GGD method was robust enough to be unaffected by the missing portions of the CFBP 6617 and WPP14 genomes. Based on the number of identities with high-scoring segment pairs (HSPs) per total HSP length, distance values of 9.8, 9.4, 10.9, 14.39 % were obtained between strain Pc1 and strains WPP14, CFBP6617, SCRI1043, and LMG20103, respectively. The distances transformed to values analogous to those of DDH (Table 2) were consistent with the proposition that strain Pc1 belongs to a different taxon than strains WPP14, CFBP6617^{T} and SCRI1043.

In a previous DNA-DNA hybridization study, Brenner *et al*. (1973) were not able to differentiate *P. aroideae* as a separate species, but then they also did not recognize *P. atrosepticum* as a separate species. The difference in results can likely be attributed to the older DNA-DNA hybridization method used, as it was later improved after further research (Brenner *et al.,* 1982). In the DNA-DNA hybridization study of Gardan *et al*. (2003), strain *P. carotovorum* subsp. *carotovorum* CFBP 1535 is one of the strains which was first reported as *E. aroideae* from *Zeae mays* and it is one of the four out-grouped strains in the data. Similarly, the pathogen reported from tobacco in the USA (Holdeman & Burholder, 1956), strain *P. carotovorum* subsp. *carotovorum* CFBP 797 (ATCC 12286), was also reported as an out-grouping strain and it, too, was initially deposited in the ATCC collection as *E. aroideae.*

E. aroideae (Townsend 1904) Holland was originally described as a *P. carotovorum* anaerogenic strain which failed to produce gas from sugars irrespective of whether or not the organism could attack arum lily (Eldor, 1941). We tested gas producing from D-glucose as described by Schaad (1988)**,** on 40 strains of *P. carotovorum,* but only strain SCRI 2 produced gas (Chapter II). Furthermore, one study reported a pathogenicity test to discriminate strains isolated from monocot plants (Seo *et al*., 2002), but we were unable to differentiate strains on the basis of pathogenicity using 40 *P. carotovorum* strains and four *P. aroideae* strains on five different host plants (radish, cucumber, pea, potato and onion) under laboratory conditions

Carbohydrate utilization by the *P. aroideae* strains was tested using the GN Biolog Gram negative microplate (GN Biolog) and conventional biochemical tests (Table S6). The data were compared to that obtained for other pectobacteria in a previous study (Chapter III). The four *P. aroideae* strains utilized D-glucuronic acid compared to <6% of strains in Yap *et al*'s (2004) study and 69 % in Gardan *et al*.'s (2003) study. *P. aroideae* utilized DL-lactic acid compared with < 20 % of *P. carotovorum* strains in a previous study (Yap *et al*., 2004), and they utilized D-galacturonic acid lactone, as did strains isolated from monocotyledonous hosts by Hu *et al*. (2007). No strains tested by Hu *et al.* (2007) utilized sorbitol or arabitol but all the *P. aroideae* strains did so**.** Costa *et al*. (2006) found one strain from apium (*Bacdonis eudicots*) which was sensitive to erythromycin but all four *P. aroideae* strains were resistant to the antibiotic.

Description of *P. aroideae* sp. nov.

Pectobacterium aroideae [a.ro.i'de.ae N.L. pl. noun aroideae pertaining to subfamily *Aroideae* (arum family) of Araceae, a family of monocotyledonous flowering plants]. Gram-negative bacterium which utilizes pectin and liquefies the semi-selective crystal violet pectate medium (CVP), is resistant to erythromycin, ferments lactose, does not produce gas from D-glucose, does not produce acid from maltose and α-methyl glucoside, does not produce reducing substances from sucrose, grows on Luria Broth with 5 % NaCl, and grows at 37 ^{o}C and 39 ^{o}C. *P. aroidea*e is distinguished from other *Pectobacterium* species in that it utilizes glycogen, β-hydroxy butyric acid, quinic acid, propionic acid, α-keto butyric acid, α-keto valeric acid, malonic acid, L-alaninamide, D-alanine, L-alanine, L-alanyl-glycine, D-serine, urocanic acid, and p-hydroxy phenylacetic, which few other strains utilize. *P. aroideae* strains are distinguished from other *P. carotovorum* strains in that they can utilize dextrin, glucuronamide, α-keto glutaric acid.

Strains of *P. aroideae* utilize carbon sources such as inosine, D,L-lactic acid, α-keto glutaric acid, D-glucuronic acid, D-glucosaminic acid, D-galactonic acid lactone, cis-aconitic acid, acetic acid, turanose, D-sorbitol, D-psicose, Tween 80, and Tween 40. *P. aroideae* displays distinctive fingerprints in ITS-PCR-RFLP analyses, having three (130, 360, 560 bp) and four (80, 120, 160, 210 bp) bands after restriction by *Rsa* I and *Hha,* respectively. Phylogenetically, *P. aroideae* clusters separately from other pectobacteria in MLSA using concatenated and individual housekeeping gene sequences (*acnA, icd, gapA, mdh, mtlD, pgi, proA* and *rpoS*). Like other pectobacteria this species also has seven 16S rDNA homologs within its chromosome and heterogeneity between the different copies. The validity of separate species status for *P. aroideae* is confirmed by DNA-DNA hybridization data and whole genome sequencing. The type strain is SCRI 109^{T} which was isolated from *Zantedeschia aethiopica* (Angoisperms, Monocots, *Alismatales*, *Araceae*, *Aroideae*, *Zentedeschia*).

5. General discussion

In this study the bacterial plant pathogen *Pectobacterium carotovorum* as the type species of the *Pectobacterium* genus was under investigation. A comprehensive study of the genetic, genomic and phenotypic characteristics of *P. carotovorum* strain population revealed two novel taxa of *P. aroideae* and *P. carotovorum* subsp. *brasiliense*. The respective study was carried out in a comparison within the six *Pectobacterium* taxa namely *P. carotovorum*, *P. atrosepticum*, *P. wasabiae*, *P. betavascularum*, *P. carotovorum* subsp. *odoriferum* and *P. carotovorum* subsp. *carotovorum*. The 76 strains investigated in this study were collected from international microbial collections and laboratories, thirty of which were isolated from Syria in the years 2002-2004.

Study the bacterial population genetics using the burgeoning technology can solve the potential difficulties of assigning new strains or species to a microbial taxon (Gevers *et al.*, 2005). The AFLP assay (Chapter II) served as a genomic screening tool demonstrated high genetic diversity existing within the *Pectobacteria* population. The AFLP- based phylogenetic analysis of fragments generated from the whole genomes demonstrated the capability of the AFLPs in differentiating *Pectobacterium* species as showed in Fig 1. These results were confirmed by the MLSA analyses results (Chapter II, III, and IV). The genome prediction based on selected genes is recently becoming a more acceptable approach in taxonomic studies (Kuhnert and Korczak, 2006**).** The MLSA data provided a confidential tool and resolved important speciation events within the *P. carotovorum* taxon as well as differentiated other *Pectobacterium* species. The MLSA sequence data were useful for differentiation at the genus, family, species and subspecies levels as confirmed by the DNA-DNA hybridization standard method for differentiating species (Chapter III and IV). Staley *et al*. (2006) confirms the utility of MLSA for additional description of family, order, class, phylum, kingdom and domain levels in the taxonomic hierarchy.

The 16S rDNA data retrieved from all species and subspecies under the genus *Pectobacterium* confirmed that the 16S rDNA does not represent the part of the genome which is useful for classification of these bacteria. However, the resolving power of the 16S rDNA has been extensively debated considering the members of the *Enterobacteriaceae* family because of the high similarity values exhibited from the 16S rDNA sequence data revealed from different *Enterobacteria* (Tindall *et al*., 2010), which was also in agreement with our findings (Chapters III and IV). The DNA-DNA association

provides a standard means for identification and classification of bacterial species, but it is inapplicable at other levels, consequently it cannot group subspecies in discrete clusters (Gevers *et al*., 2005). However, the study faced limitation in the Lacked platform or capability to suggest additional taxa to the subdivisions revealed from the phylogeny of both *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliense*, even this was clear by phenotypic criteria (chapter II). However, the DNA-DNA association method is a very laborious technique and only a limited number of laboratories in the world possess the standards needed to achieve convenient results, which makes the use of the DNA-DNA hybridization very limited. We are grateful to the scientific services offered by the DSMZ who performed this analysis. In chapter IV we made use of the available *in-silico* DNA-DNA association methods based on the available genome sequencing of four *Pectobacterium* strains (Pc1, SCRI1043, WPP14, Pcbr 212). As expected, the results obtained from the *in-silico* hybridization were comparable to the lab method.

In this study, we focus on the plant pathogen *P. carotovorum* considering its dilemma of an unjustified high heterogeneity in the species of pectolytic bacteria. However, some pectolytic species like *P. atrosepticum* are well identified bacteria, the latter exhibiting a high homogeneity among the strains, as confirmed by the AFLP profile and MLSA sequence data, independent of the geographical source of the strains exemplified by the seven *P. atrosepticum* strains identified in chapter II. The strains of the *P. atrosepticum* species are highly genetically identical, as well as it is still the only *Pectobacterium* specie holding host specificity to potato (*Solanum tuberosum* L.) (Helias *et al*., 1998). The species *P. wasabiae* shows a specific genomic organisation and phenotypic criteria and has expanded its definition from being a pathogen that causes soft rot disease on horseradish plants and some vegetable crops especially in Japan to a major blackleg disease on potato with almost worldwide distribution (Pitman *et al*., 2009). This extension in the host range of *P. wasabiae* accompanied by genetic justifications will probably lead to the establishment of more infra-subdivisions. The MLSA sequence data of *P. wasabiae* strains isolated from potato displayed differences in the conservative genes compared to the strains isolated from horseradish, pointing at the need for more dedicated studies to find out the genetic correlation among *P. wasabiae* strains and the relevant hosts (Ma *et al*., 2007). Our study re-identified strains of *P. carotovorum* as *P. wasabiae* (Chapter II), showing that these extended host range is not only newly detected, but occurred already a

long time ago even before the *P. wasabiae* species was established and identified in Japan, in year 1987 'as information retrieved from the isolation date of the strains which re-identified in this study as *P. wasabiae*'.

Throughout previous studies isolated pectolytic pathogens, *P. carotovorum* comprised strains which could not be assigned to any other *Pectobacterium* species with more certain identifications. It was clarified based on the DNA-DNA hybridization in chapter IV that *P. carotovorum* harbours at least two confirmed species of *P. carotovorum* and *P. aroideae.* The previous study of Brenner *et al*. (1973) based on DNA-DNA association did not differentiate *P. aroideae* as a separate species as well as did not recognize *P. atrosepticum*. However, different reasons can explain these results: first, the DNA-DNA association method was not well adjusted and Brenner himself improved the method in 1982. Furthermore, some of the strains representing each species and used for the hybridization study may have been miss-identified based on the available physiological criteria at the time of Brenner's study (1973).

P. carotovorum clearly divided into three subspecies, *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum* and *P. carotovorum* subsp. *brasiliense* on the basis of MLSA sequence data, AFLP fragment patterns as well as 16S rDNA provided in chapter III. Moreover, two infra-subdivisions are resolved for each *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *brasiliense* in chapter II and IV and need more justifications using the whole genome sequencing comparison projects (Glasner *et al*., 2009). The DNA sequencing guarantees unambiguous information, high resolution, and has the advantage of providing electronic portable data with an accumulation facility when more genomes are sequenced and available for more electronic pairing (Auch *et al*., 2010b). The genome sequencing can provide a solution for infra-subspecies and subspecies which cannot be resolved by DNA-DNA hybridization. However, the case of infra-subspecies is not yet accepted as a taxonomic level (Bull *et al*., 2008).

The subspecies *P. carotovorum* subsp. *odoriferum* studied in chapter III in comparison to other *P. carotovorum* and confirmed as a very homogeneous taxon. *P. carotovorum* subsp. *odoriferum* strains produced their own phylogenetic groups based on the different regions of the genome used in this study of the eight housekeeping genes (*acnA*, *proA*, *mdh*, *gapA*, *mtlD*, *rpoS*, *icd*, *pgi*), the 16S rDNA sequence data, and the AFLP pattern computations (Avrova *et al*., 2002). Strains of *P. carotovorum* subsp.

odoriferum have a very limited host range and geographical distribution and were isolated only in Europe (Gallois *et al*., 1992). Since this subspecies is not a potato pathogen, this could explain the low frequency in isolating strains of this pathogen which cause the soft rot disease on chicory, a crop which is not as economically important as much as potato. The *P. carotovorum* subsp. *odoriferum* as a separate taxon has been debated in phylogenetic studies, confirming that using more than one strain is very significant factors in phylogenetic studies, that would be even truer in case of bacterial species or subspecies which belong to high divergent taxa such as *P. carotovorum* (Ma *et al*. 2007).

This study assigned additional strains from different geographical sources and additional host plants to the taxon *P. carotovorum* subsp. *brasiliense* as part of the strain collection (Chapter II and III). The *P. carotovorum* subsp. *brasiliense* name has now become legitimated upon the published data from chapter III of the 16S rRNA and DNA-DNA association in the International Journal of Systematic and Evolutionary Microbiology. The study showed that the number of strains assigned to this taxon is dramatically increasing. Even though this taxon reduces the problematic heterogeneity of the *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *brasiliense* itself becomes more questionable. The data of the AFLP, MLSA and the phenetic criteria displayed unexpected heterogeneity among the strains. The geographically discriminated two clades of *P. carotovorum* subsp. *brasiliense* need further investigation (Chapter II).

It is evident that some of the differential phenotypic criteria classically used to identify *Pectobacteria* are not consistently useful for classifying currently recognized species and subspecies (Chapter II, III and IV) (Gallois *et al*., 1992: Duarte *et al*., 2004). Both *P. carotovorum* subsp. *brasiliense* and *P. carotovorum* subsp. *odoriferum* were initially identified as atypical *P. atrosepticum* (Gallois *et al*., 1992: Duarte *et al*., 2004). Additionally, one unstable criterion of calling the *P. aroideae* a non-gas-forming isolate of *P. carotovorum* contributed to misidentifying both pathogens (Graham *et al*., 1964). Meanwhile, these intermixed phenotypic criteria between the two species *P. carotovorum* and *P. aroideae* prevent using the correct strains for differentiating studies, resulting in the decision of omitting the *P. aroideae* species and in considering the previously to this species assigned strains as part of the heterogeneous pool of the species *P. carotovorum*.

P. carotovorum was described as a pathogen that causes soft rot disease on tubers, bulbs, rhizomes and leaves of different ornamental plants including *Arum lily*, *Allium cepa,*

Zantedeschia spp., *Ornithogalum* spp., *Dieffenbachia* spp., *Pinellia ternata, Amorphophallus konjac,* etc. and has been isolated from ornamental crop plants in many countries such as Korea, Japan, the Netherlands, China, India, and recently in Iran (Smith and Bartz, 1990; Wright *et al*, 2001; Snijder and Tuyl, 2002; Hahm *et al* 2003; Norman *et al*., 2003; Snijder *et al*., 2004; Hu *et al*., 2007; Baghaee-Ravari *et al*., 2010). In this context, these studies were likely describing *P. aroideae*, which can also infect tobacco (Xia *et al*., 2007) and other dicotyledonous plants, but with a lower probability. In our strain collection (Chapter II), the two strains isolated from monocotyledonous plants were belonging to the new species *P. aroideae,* as well as two strains retrieved from the Genbank and isolated from monocotyledonous plants.

The case of the *P. aroideae* species is the lonely recent success in studies addressing host specificity of pectolytic bacteria. This species was considered before as *Erwinia aroideae* (Townsend) Holland (1904). A long debate lasts about 70 years from the first naming of *Erwinia aroideae* until the decision taken to remove the name from the biological code of bacteria. Consequently, the species name *Erwinia aroideae* disappears from the eighth edition of Bergey's Manual. We revive the epithet *aroideae* and stimulate it according to the new nomenclature of the genus *Pectobacterium* to be *P. aroideae.* About 60 % of strains isolated from monocotyledonous plant families are expected to be belonging to this species (Yishay *et al*., 2008). However, strains isolated from dicotyledonous plant families are also entitled under the host range of this pectolytic plant pathogen, but with low probability, as shown in the phylogenetic cluster PcV (chapter II).

A comparison of GN Biolog based data from different *P. carotovorum* studies demonstrated a high level of variation in carbon source utilization among strains (Chapters III and IV). The four *P. aroideae* strains utilized D-glucuronic acid compared to < 6% of strains in Yap *et al.*'s (2004) study and 69% in Gardan *et al.*'s (2003) study. *P. aroideae* utilized DL-lactic acid compared with < 20 % of *P. carotovorum* strains in a previous study (Yap *et al.* 2004), and they utilized D-galacturonic acid lactone, as did strains isolated from monocotyledonous hosts described by Hu *et al*. (2007). No strain tested by Hu *et al.* (2007) utilized sorbitol or arabitol, but all the *P. aroideae* strains did so. Costa *et al*. (2006) found one strain from apium (*Bacdonis eudicots*), which was sensitive to erythromycin but all four *P. aroideae* strains were resistant to the antibiotic. Thus, these shared physiological data did not help in following the similar strains in different studies.

Kim *et al*. (2009) suggested that RFLP analysis is no longer reliable, especially at the level of *P. carotovorum* species. However, in another study (Zhu *et al*., 2010) it was suggested that two additional specific banding profiles, which were not described in the first study of Toth *et al*. (2001), should additionally assign *Pectobacterium* strains to *P. carotovorum* species. One of the two banding profiles obtained by Zhu *et al*. (2010) for strain D4 (isolated from Chinese cabbage) is identical to the banding patterns obtained from *P. aroideae* strains in chapter IV which can confirm this strain as belongs to the *P. aroideae*.

6. General conclusion

The study comprises a wide range of methodological approaches for differentiation of *Pectobacteria* and has resulted in significant progress for their taxonomy. Both taxa namely *P. carotovorum* subsp. *brasiliense* and *P. aroideae* reported in this study contribute to reduce the heterogeneity within strains belong to the *P. carotovorum* species. Additional speciation events are suggested based on the phylogenetic analyses performed in this study. Strains such as C336.1 and C331 cannot be unequivocally placed into any of the present *Pectobacterium* taxa.

It is important that the identification of the old and new strains deposited in international collections and laboratories is accurate. Huge efforts have been lost because they based on mis-identified strains, exemplified by the only one available whole genome sequence of the bacterial plant pathogen *P. carotovorum* strain Pc1, the strain belonging to the new species *P. aroideae* as confirmed in our study. In the thesis of Ahmed (2002) the plant pathogen *P. carotovorum* strain GSPB 436 was used as the model, while this strain was re-identified as belonging to the genus *Dickeya*.

The plant pathogen *Pectobacterium* is in a constant state of genetic exchanges with closely related species and subspecies, as well as with other organisms in the environment. The clear sensibility of these pathogens to genetic manipulation necessitates a continuous evaluation and comparison among strains from different sources and time points using the burgeoning technology of sequencing. The description of microbial species established in the nineteen century such as the *P. carotovorum* has to be re-evaluated as well.

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Transformed *E. coli* colonies (clones) were numbered as clone 1, 2, 3, 4 and transferred into 2.5 ml LB+ Ampicillin medium in 30 ml glass tubes.

Plasmid extraction:

1- Grow the clones in a shaking incubator for 4-5 h, at 73 ^{o}C with 180 rpm.
2- Transfere the cultures to 2.0 ml eppendorfs and harvest the cells by centrifugation for 90 s at 10.000 rpm.
3- Resuspend the cell pellets in 200 µl solution A, add 400 µl solution B, 300 µl solution C, gently mix after adding each solution.
4- Incubate the tubes for 30 min on ice. After incubation, centrifuge for 10 min at 13.000 rpm.
5- Transfere the supernatants to a new 1.5 ml eppendorfs.
6- Repeat step 5 (G. 10´, 13.000 rpm), and again transfer the supernatant to new 1.5 ml eppendorfs.
7- Add 600 µl isopropanol (-20 ^{o}C), shack.
8- Centrifuge for 10 min at 13000 rpm, through supernatants.
9- Add 200 µl solution D and incubate for 5 min allowing pellets to elute properly in the solution.
10- Add 400 µl Eth. (100 %) (Eth. stored at -20 ^{o}C).
11- Centrifuge for 10 min at 13.000 rpm, through supernatant properly.
12- Using vacuum drier, dry the pellet for 10 min.
13- Add 80 µl TE-RNase (10 µl RNase in 10 ml TE)

Solution A: (500 ml)

- 15 mM Tris
- 10 mM EDTA
- 50 mM Glucose

PH. 8.0, autoclave for 15 min

Solution B: (500 ml)

- 0.2 M NaOH
- 1% SDS

autoclave for 15 min

Solution C: (500 ml)

- 3 M NaAc

PH. 5.2, autoclave for 15 min

Solution D: (500 ml)

- 10 ml solution 1 (0.5 M Tris, PH. 8.0 + HCl)
- 10 ml solution 2 (1 M NaAc, PH. 7.0)
- Fill up to 100 Bidest H2O, Autoclave 15 min.

Table S1.

Strains included as electronic data clustered in the revealed clades having the designation from the GenBank.

Clusters		Strains designation
P. carotovorum	PcI	97, 380, WPP14, WPP220, WPP221, WPP236
	PcII	21, 63, 71, 193
	PcIII	SCRI482
	PcIV	46, 87, 105, 938, 939, 940, 1692, 1695, WPP17, WPP165
	PcV	106, 1
P. atrosepticum		6, 8, SCRI1043
P. betavasculorum		1, 2, 6
P. wasabiae		SCRI488, WPP19, WPP161, WPP163, WPP168, WPP172
Dickeya spp.		*D.da*586, *D.da*646, *D.da*678, *D.da*721, *D.da*0862, *D. zeae* 1591

Table S2.

Strains that did not cluster properly in the ML trees computed separately for individual genes (Figures S2 to S9).

Clusters		***acnA***	***rpoS***	***mtlD***	***icd***	***pgi***	***mdh***	***proA***	***gapA***
P. carotovorum	Pc I	*P.ba*6 C193 C142.1b	X	X	*P.ba*6	X	SCRI121 WPP17		X
P. carotovorum	Pc II	C3		WPP17 C338 M30 N78 C393.1 C317.1			X		X
P. carotovorum	PcIII		C140.2 C144	X	*D.*spp436	M30			X
P. carotovorum	PcIV					*D.da*586			SCRI3
P. carotovorum	Pc V							C317.1 SR260	
P. atrosepticum		JKI4.3.14 C150	SCRI121 C412.4, *D.*spp436			SCRI121	JKI4.3.14		
P. wasabiae									
P.betavascularum							X		SCRI1043
***Dickeya* spp.**					JKI568				

The sequenced *acnA* gene portion from the strain *Dickeya* spp.SR260, showed the same data as obtained from the strain *Erwinia stewartii* LMG2715, which was identified only as an out group.
X: the cluster could not be produced.

Table S3.

Table S3.1. *Pectobacterium and Dickeya* strain designation and Genebank accession numbers for the eight genes: *acnA, gapA, icdA, mdh, mtlD, pgi, proA,* and *rpoS* of 63 strains in chapter I.

Strain designation	GenBank accession numbers							
	acnA	***gapA***	***icdA***	***mdh***	***mtlD***	***pgi***	***proA***	***rpoS***
A6.2	HM156760	HM156820	HM156881	HM156942	HM157004	HM157066	HM157128	HM157190
A10.1	HM156761	HM156821	HM156882	HM156943	HM157005	HM157067	HM157129	HM157191
A10.2	HM156762	HM156822	HM156883	HM156944	HM157006	HM157068	HM157130	HM157192
A16	HM156763	HM156823	HM156884	HM156945	HM157007	HM157069	HM157131	HM157193
A17	HM156764	HM156824	HM156885	HM157071	HM157008	HM157070	HM157132	HM157194
A18	HM156765	HM156825	HM156886	HM156947	HM157009	HM157071	HM157133	HM157195
A45	HM156766	HM156826	HM156887	HM156948	HM157010	HM157072	HM157134	HM157196
C3	HM156767	HM156827	HM156888	HM156949	HM157011	HM157073	HM157135	HM157197
C18	HM156768	HM156828	HM156889	HM156950	HM157012	HM157074	HM157136	HM157198
C137	HM156769	HM156829	HM156890	HM156951	HM157013	HM157075	HM157137	HM157210
C140.2	HM156770	HM156830	HM156891	HM156952	HM157014	HM157076	HM157138	HM157211
C142.1b	HM156771	HM156831	HM156892	HM156953	HM157015	HM1570677	HM157139	HM157212
C142.1s	HM156772	HM156832	HM156893	HM156954	HM157016	HM157078	HM157140	HM157213
C142.2	HM156773	HM156833	HM156894	HM156955	HM157017	HM157079	HM157141	HM157214
C143	HM156774	HM156834	HM156895	HM156956	HM157018	HM157080	HM157142	HM157215
C144	HM156775	HM156835	HM156896	HM156957	HM157019	HM157081	HM157143	HM157216
C150	HM156776	*	HM156897	HM156958	HM157020	HM157082	HM157144	HM157217
C267	HM156777	HM156836	HM156898	HM156959	HM157021	HM157083	HM157145	HM157218
C317.1	HM156778	HM156837	HM156899	HM156960	HM157022	HM157084	HM157146	HM157219
C331	HM156779	HM156838	HM156900	HM156961	HM157023	HM157085	HM157147	HM157220
C338	HM156780	HM156839	HM156901	HM156962	HM157024	HM157086	HM157148	HM157221
C364.2	HM156781	HM156840	HM156902	HM156963	HM157025	HM157087	HM157149	HM157222
C380	HM156782	HM156841	HM156903	HM156964	HM157026	HM157088	HM157150	HM157223
C393.1	HM156783	HM156842	HM156904	HM156965	HM157027	HM157089	HM157151	HM157226
C412.4	HM156784	HM156843	HM156905	HM156966	HM157028	HM157091	HM157152	HM157227
M30	HM156785	HM156844	HM156906	HM156967	HM157029	HM157099	HM157153	HM157199
N78	HM156786	HM156845	HM156907	HM156968	HM157030	HM157098	HM157154	HM157200
SCRI2	HM156788	HM156846	HM156908	HM156969	HM157031	HM157104	HM157155	HM157202
SCRI3	HM156789	HM156847	HM156909	HM156970	HM157032	HM157105	HM157156	HM157203
SCRI1073	HM156787	HM156848	HM156910	HM156971	HM157033	HM157090	HM157157	HM157201

Appendix: Supplementary tables

JKI4.3.8	HM156790	HM156849	HM156911	HM156972	HM157036	HM157092	HM157158	HM157204
JKI4.3.14	HM156791	HM156850	HM156912	HM156973	HM157038	HM157093	HM157159	HM157205
JKI4.3.22	HM156792	HM156851	HM156913	HM156974	HM157035	HM157094	HM157160	HM157206
SCRI102	HM156793	HM156852	HM156914	HM156975	HM157034	HM157095	HM157161	HM157207
SCRI109	HM156794	HM156853	HM156915	HM156976	HM157037	HM157096	HM157162	HM157208
SCRI121	HM156795	HM156854	HM156916	HM156977	HM157039	HM157097	HM157163	HM157224
SCRI132	HM156796	HM156855	HM156917	HM156978	HM157040	HM157100	HM157164	HM157209
JKI568	HM156797	HM156856	HM156918	HM156979	HM157041	HM157101	HM157167	HM157228
JKI582	HM156799	HM156857	HM156919	HM156980	HM157042	HM157102	HM157165	HM157229
NB1892	HM156798	HM156858	HM156920	HM156981	HM157043	HM157103	HM157166	HM157230
SCRI1071	HM156605	HM156865	HM156921	HM156988	HM157050	HM157112	HM157171	HM157236
JKI17A-1	HM156802	HM156860	HM156922	HM156984	HM157048	HM157108	HM157170	HM157234
JKI4.2.5	HM156800	HM156864	HM156923	HM156983	HM157045	HM157106	HM157168	HM157231
M37	HM156804	HM156861	HM156924	HM156985	HM157047	HM157111	HM157174	HM157237
JKI185	HM156803	HM156863	HM156925	HM156987	HM157049	HM157110	HM157169	HM157235
SCRI8	HM156801	HM156859	HM156926	HM156982	HM157046	HM157107	HM157173	HM157232
JKI4.2.9	HM156806	HM156862	HM156927	HM156986	HM157044	HM157109	HM157172	HM157233
NB2122	HM156810	HM156866	HM156928	HM156992	HM157054	HM157113	HM157178	HM157241
SCRI207	HM156807	HM156868	HM156929	HM156991	HM157051	HM157115	HM157177	HM157239
JKI4.2.6	HM156808	HM156869	HM156930	HM156989	HM157053	HM157114	HM157176	HM157240
JKI1A-1	HM156809	HM156867	HM156931	HM156990	HM157052	HM157116	HM157175	HM157238
JKI4.9.3	HM156812	HM156872	HM156932	HM156993	HM157057	HM157117	HM157179	HM157242
GSPB436	HM156811	HM156870	HM156933	HM156998	HM157059	HM157121	HM157182	HM157225
JKI4.9.4	HM156813	HM156871	HM156934	HM156996	HM157058	HM157118	HM157183	HM157243
JKI4.9.22	HM157252	HM156873	HM156935	HM156997	HM157060	HM157119	HM157184	HM157244
C89.1	HM156814	HM156875	HM156936	HM156994	HM157055	HM157122	HM157180	HM157251
C89.2	HM156815	HM156876	HM156937	HM156995	HM157056	HM157123	HM157181	HM157250
GSPB4610	HM156816	HM156877	HM156938	HM157000	HM157062	HM157124	HM157186	HM157246
GSPB30177	HM156817	HM156878	HM156939	HM157001	HM157063	HM157125	HM157187	HM157247
GSPB30178	HM156818	HM156879	HM156940	HM157002	HM157064	HM157126	HM157188	HM157248
GSPB30179	HM156819	HM156880	HM156941	HM157003	HM157065	HM157127	HM157189	HM157249
SR260	HM157253	HM156874	*	HM156999	HM157061	HM157120	HM157185	HM157245

* Missing data

BLAST similarity search resulted for HM157252 (*acnA*, strain *Dickeya* sp. 4.9.22), indicates more similarity to aconitate hydratase 2 gene than to supposed aconitate hydratase 1.

Table S3.

Table S3.2. *Pectobacterium* strain designation and Genebank accession numbers for the nine genes: *acnA, gapA, icdA, mdh, mtlD, pgi, proA,* and *rpoS* of strains in chapter III and chapter IV.

Strain designation	GenBank accession numbers							
	acnA	***gapA***	***icdA***	***mdh***	***mtlD***	***pgi***	***proA***	***rpoS***
CFBP2046	JF926762	JF926772	JF926782	JF926792	JF926802	JF926812	JF926822	JF926833
CFBP1878	JF926763	JF926773	JF926783	JF926793	JF926803	JF926813	JF926823	JF926834
1033	JF926764	JF926774	JF926784	JF926794	JF926804	JF926814	JF926824	JF926835
1034	JF926765	JF926775	JF926785	JF926795	JF926805	JF926815	JF926825	JF926836
1009	JF926766	JF926776	JF926786	JF926796	JF926806	JF926816	JF926826	JF926837
1001	JF926767	JF926777	JF926787	JF926797	JF926807	JF926817	JF926827	JF926838
8	JF926768	JF926778	JF926788	JF926798	JF926808	JF926818	JF926828	JF926839
212	JF926769	JF926779	JF926789	JF926799	JF926809	JF926819	JF926829	JF926840
371	JF926770	JF926780	JF926790	JF926800	JF926810	JF926820	JF926830	JF926841
213	JF926771	JF926781	JF926791	JF926801	JF926811	JF926821	JF926831	JF926842
CFBP1526	JN600333	JN600336	JN600339	JN600342	JN600345	JN600348	JN600351	JN600354
CFBP2122	JN600334	JN600337	JN600340	JN600343	JN600346	JN600349	JN600352	JN600355
CFBP3304	JN600335	JN600338	JN600341	JN600344	JN600347	JN600350	JN600353	JN600356

Table S3.

Table S3.3. *Pectobacterium* strain designation and Genebank accession numbers for 16S rDNA homologs of strains in chapter III and chapter IV.

Strain designation	Homologs	GenBank accession numbers
JKI582	I	JF926729
	II	JF926730
CFBP2046	I	JF926731
	II	JF926732
SCRI2	I	JF926733
	II	JF926734
A10.1	I	JF926735
	II	JF926736
A18	I	JF926737
	II	JF926738
C144	I	JF926739
	II	JF926740
C3	I	JF926741
	II	JF926742
C150	I	JF926743
	II	JF926744
C267	I	JF926745
	II	JF926746
C137	I	JF926747
	II	JF926748
C412.4	I	JF926749
	II	JF926750
C142.2	I	JF926751
	II	JF926752
C338	I	JF926753
	II	JF926754
M30	I	JF926755
	II	JF926756
JKI4.3.8	I	JF926757
	II	JF926758
1001	I	JF926759
	II	JF926760
SCRI3	I	JN600320
	II	JN600321
SCRI109	I	JN600322
	II	JN600323
SCRI121	I	JN600324
	II	JN600325
SCRI102	I	JN600326
	II	JN600327
CFBP2122	I	JN600328
	II	JN600329
CFBP 3304	I	JN600330
	II	JN600331
212	I	JF926716
	II	JF926761
1073	I	JF926717
C18	I	JF926718
	II	JF926719
1033	I	JF926720
A17	I	JF926721
	II	JF926722
8	I	JF926723
	II	JF926724
317	I	JF926725
	II	JF926726
CFBP1878	I	JF926727
NB1892	I	JF926728

Table S4.

Primers used for 16S rDNA amplification and sequencing.

Primer name	Sequence (5`→ 3`)	Reference	Used for
Lpf	GGGCACTCACAAGACCGTAT	This study	amplification
Rpr	GACAATCTGTGTGAGCACTTCA	This study	amplification
S523F	GTAATACGGAGGGTGCAAGC	This study	sequencing
S1024F	CTGTGAGACAGGTGCTGCAT	This study	sequencing
G1-rev	CCTTGTTACGACTTC	Fessehaie *et al*. (2002)	sequencing
907-rev	CCGTCAATTCATTTGAGTTT	Fessehaie *et al*. (2002)	sequencing
Dr-rev	GTGCAATATTCCCCACTGCT	This study	sequencing
R	Provided with the kit	Vector primer	sequencing
F	Provided with the kit	Vector primer	sequencing

Table S5.

Number and identity of base substitution (A, C, G, T) at each parsimony informative site in the 1530 bp of the16S rDNA gene in each of the three subspecies of *P. carotovorum*, *Escherichia coli* 16S rDNA gene sequence numbering is indicated.

	Nucleotide site	Consensus base	*P.c.* subsp. *brasiliensis* n. 16	*P.c.* subsp. *odoriferum* n. 6	*P.c.* subsp. *carotovorum* n. 28
1	65	A	2 G	-	-
2	76	A	2 G	-	-
3	77	G	-	3 A	-
4	78	A	2 G	6 G	4 G
5	79	A	1 G	-	16 G
6	90	T	1 C	-	16 C
7	91	T	2 C	6 C	3 C
8	210	C	2 A	-	7 A
9	445	G	-	6 A	28 A
10	456	G	4 A	6 A	-
11	457	G	1 T	-	3 A
12	458	G	9 A	6 C	27 A
13	459	A	-	6 G	-
14	460	A	-	6 G	-
15	461	G	1 A	6 T	1 A
16	469	A	-	6 G	-
17	471	C	3 T	6A	-
18	472	T	-	6 C	-
19	473	T	5 C	6 G	1 G
20	474	T	2 G+1 C	6 G	-
21	475	C	2 C	6 C	-
22	476	C	3 T	6 T	-
23	489	C	-	6 T	28 T
24	1001	G	2 C	-	12 C
25	1006	T	11 C	-	4 C
26	1008	T	2 C	6 A	9 C+5 A
27	1009	G	11 A	-	4 T
28	1010	C	13 G	-	13 G
29	1011	T	13 C	-	4 C
30	1018	A	13 G	-	4 G
31	1019	G	13 C	-	13 C
32	1020	C	11 T	-	4 A
33	1022	T	-	6 G	5 G
34	1023	A	11G	-	4 G
35	1039	A	2 G	-	12 G

Sequence types which were identical are:

Sequences obtained from *P.c.*subsp. *brasiliensis* 1033 were identical to one sequence type of strain A17.

Sequences obtained from *P.c.*subsp. *odoriferum* type strain were identical to one sequence type of JKI582.

Sequences obtained from NB1892 were identical to one sequence type of strain JKI582.

One sequence type obtained from *P.c.*subsp.*carotovorum* type strain was identical to one sequence of SCRI2.

One sequence type obtained from *P.c.*subsp.*carotovorum* type strain was identical to one sequence of A18 and one sequence of A10.1.

One sequence type obtained from *P.c.*subsp.*carotovorum* A10.1 was identical to one sequence of C144.

One sequence type obtained from *P.c.*subsp.*carotovorum* C150 was identical to one sequence of C142.2 and one sequence of C267.

One sequence type obtained from *P.c.*subsp.*carotovorum* C150 was identical to one sequence of JKI 4.3.8.

One sequence type obtained from *P.c.*subsp.*carotovorum* C412.4 was identical to one of JKI4.3.8.

Table S6.

Strains designation, CFBP and DSM accession numbers.

CFBP: French collection of plant pathogenic bacteria.

DSM: German resource centre for biological material

Strain designation	CFBP numbers	DSM numbers
P. carotovorum subsp. *carotovorum* A6.2	7346	3891
P. carotovorum subsp. *carotovorum* A10.1	7347	
P. carotovorum subsp. *carotovorum* A10.2	7348	
P. carotovorum subsp. carotovorum A18	7350	
P. carotovorum subsp. *carotovorum* A16	7349	
P. carotovorum subsp. *carotovorum* M30	7356	
P. carotovorum subsp. *carotovorum* N78	7351	
P. carotovorum subsp. *carotovorum* C140.2	7353	
P. carotovorum subsp. *carotovorum* C144	7367	
P. carotovorum subsp. *carotovorum* C338	7354	
P. carotovorum subsp. *carotovorum* C3	7352	3893
P. carotovorum subsp. *carotovorum* C380	7355	
P. carotovorum subsp. *carotovorum* C412.4	7372	
P. carotovorum subsp. *carotovorum* C137	7362	
P. carotovorum subsp. *carotovorum* C143	7366	
P. carotovorum subsp. *carotovorum* C150	7368	3894
P. carotovorum subsp. *carotovorum* C142.1b	7363	
P. carotovorum subsp. *carotovorum* C142.2	7365	
P. carotovorum subsp. *carotovorum* C142.1s	7364	
P. carotovorum subsp. *carotovorum* C267	7369	3897
P. carotovorum C331	7370	
P. carotovorum C364.2	7371	3890
P. carotovorum subsp. *brasiliense* C317.1	7360	
P. carotovorum subsp. *brasiliense* C393.1	7361	
P. carotovorum subsp. *brasiliense* C18	7359	
P. carotovorum subsp. *brasiliense* A45	7358	3892
P. carotovorum subsp. *brasiliense* A17	7357	
P. atrosepticum M37	7375	3895
Dickeya spp. C89.1	7373	
Dickeya spp. C89.2	7374	23896

Table S7. Physiological criteria of strains of soft rot *Pectobacterium* and *Dickeya*.

	substrates	***P.a* n. 7**	***P.cc* n. 23**	***P.co* n. 3**	***P.cbr* n. 8**	***P.atr* n. 4**	***P. beta n. 1***	***P.w* n. 3**	***Dickeya* n. 12**
1	Dextrin	2	2	0	1	4	0	2	6
2	Glycogen	1	1	0	1	4	0	1	3
3	Tween 40	2	3	1	1	4	0	3	7
4	Tween80	8	7	3	2	4	1	3	10
5	L-Fucose	2	4	0	2	3	0	2	9
6	Xylitol	0	2	1	1	3	0	1	3
7	Maltose	2	8	3	2	4	0	2	9
8	D-Psicose	7	11	3	3	4	1	3	12
9	D-Sorbitole	2	3	3	2	4	0	2	10
10	Turanose	2	2	1	2	4	0	2	6
11	Acetic Acid	6	9	0	1	4	0	3	8
12	Cis-Aconitic Acid	3	3	0	2	4	0	3	12
13	D-Galactonic Acid Lactone	7	13	1	7	4	1	3	8
14	D-Glucosaminic Acid	7	4	0	2	4	0	3	12
15	D-Glucuronic Acid	1	4	0	2	4	0	2	3
16	β- Hydroxy Butyric Acid	1	1	0	1	3	0	1	2
17	Glucuronamide	2	3	0	1	4	0	1	5
18	Quinic Acid	0	1	0	2	3	0	1	2
19	Propionic Acid	1	1	0	1	3	0	1	1
20	α-Keto Butyric Acid	0	0	0	0	2	0	0	2
21	α-Keto Glutaric Acid	6	2	0	1	4	0	3	7
22	α-Keto Valeric Acid	0	0	0	0	2	0	0	1
23	D,l-Lactic Acid	7	10	0	1	4	1	3	12
24	Malonic Acid	1	0	0	2	3	0	1	10
25	L-Alaninamide	0	0	0	1	3	0	1	5
26	D-Alanine	2	2	0	1	3	0	1	3
27	L-Alanine	2	2	0	2	4	0	1	5
28	L-Alanyl-glycine	2	1	0	2	3	0	1	3
29	D-Serine	1	3	0	2	3	0	1	2
30	Inosine	7	8	1	3	4	1	2	6
31	Urocanic Acid	1	2	0	2	3	0	1	2
	p-Hydroxy Phenylacetic	0	0	0	0	2	0	0	1

Gram Negative Biolog data of 14 *P. carotovorum* subsp. *carotovorum*, three *P. carotovorum* subsp. *odoriferum*, and eight *P. carotovorum s*ubsp. *brasiliense* were obtained from chapter III, Nine *P. carotovorum*, seven *P. atrosepticum*, three *P. wasabiae*, one *P. betavasculorum* and 12 *Dickeya* ssp. strains are the results of chapter IV.

Figure S1.

Evolutionary relationships of 63 taxa inferred using the Maximum likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The ML dendrogram generated using eight concatenated housekeeping gene sequences represents the evolutionary relationships among 51 strains of *Pectobacterium* spp. and 12 strains of *Dickeya* spp.

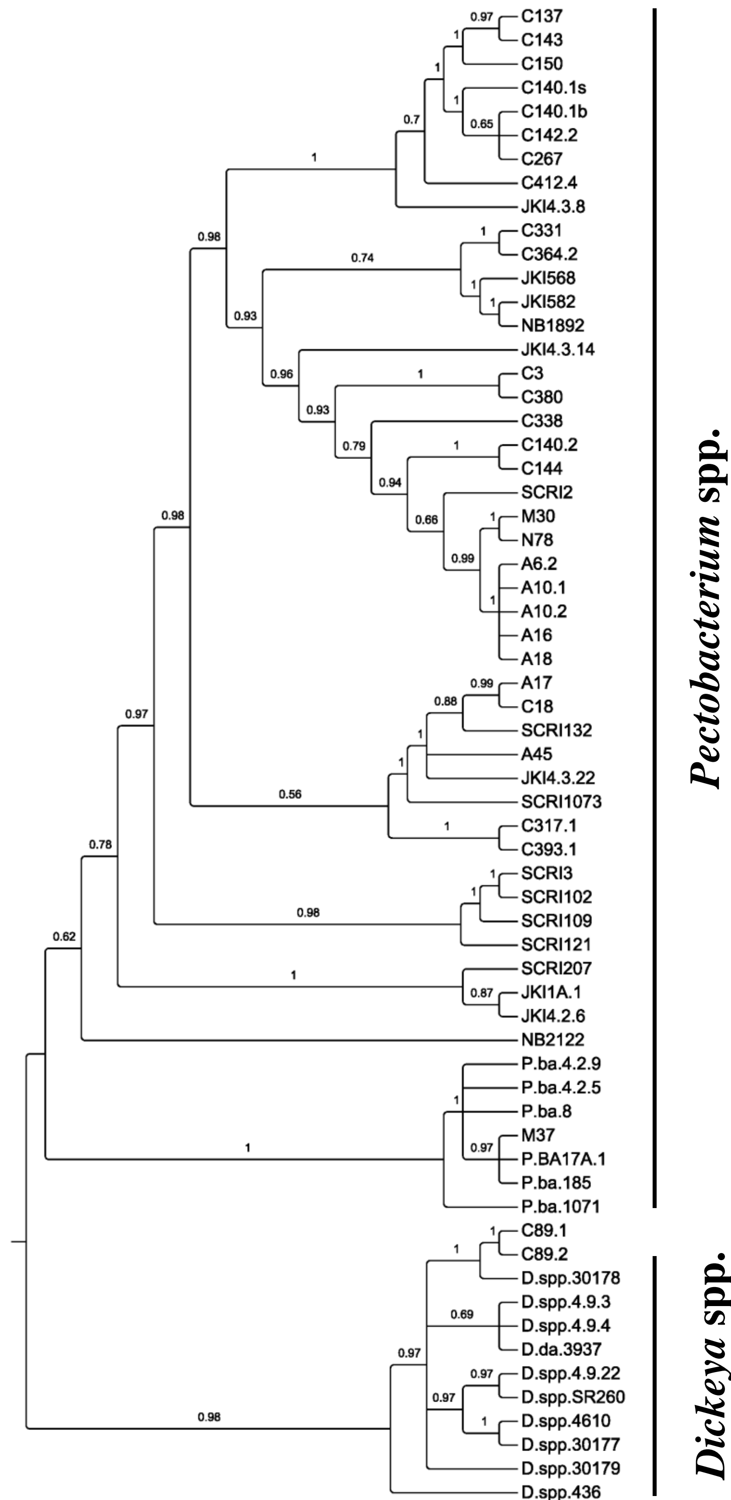


Figure S2.

ML individual tree computed for the *ProA* gene fragments of 104 taxa.

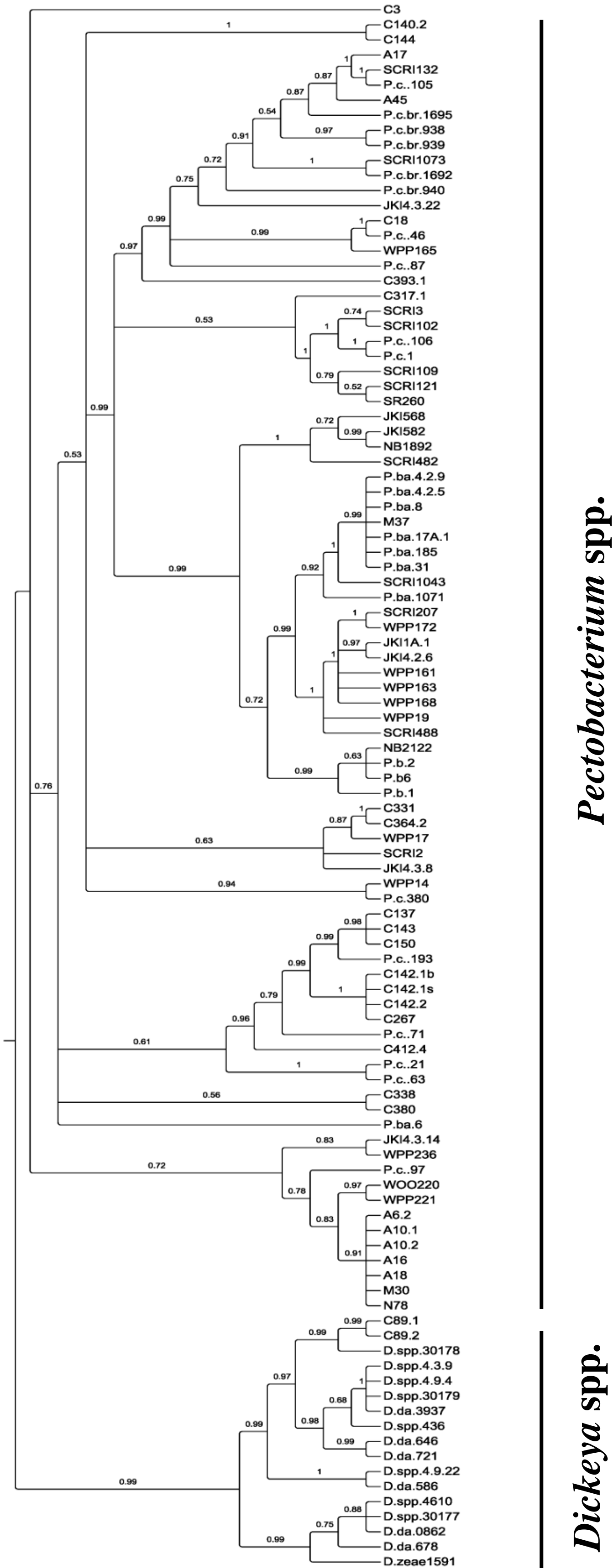


Figure S3.

ML individual tree computed for the *gapA* gene fragments of 104 taxa.

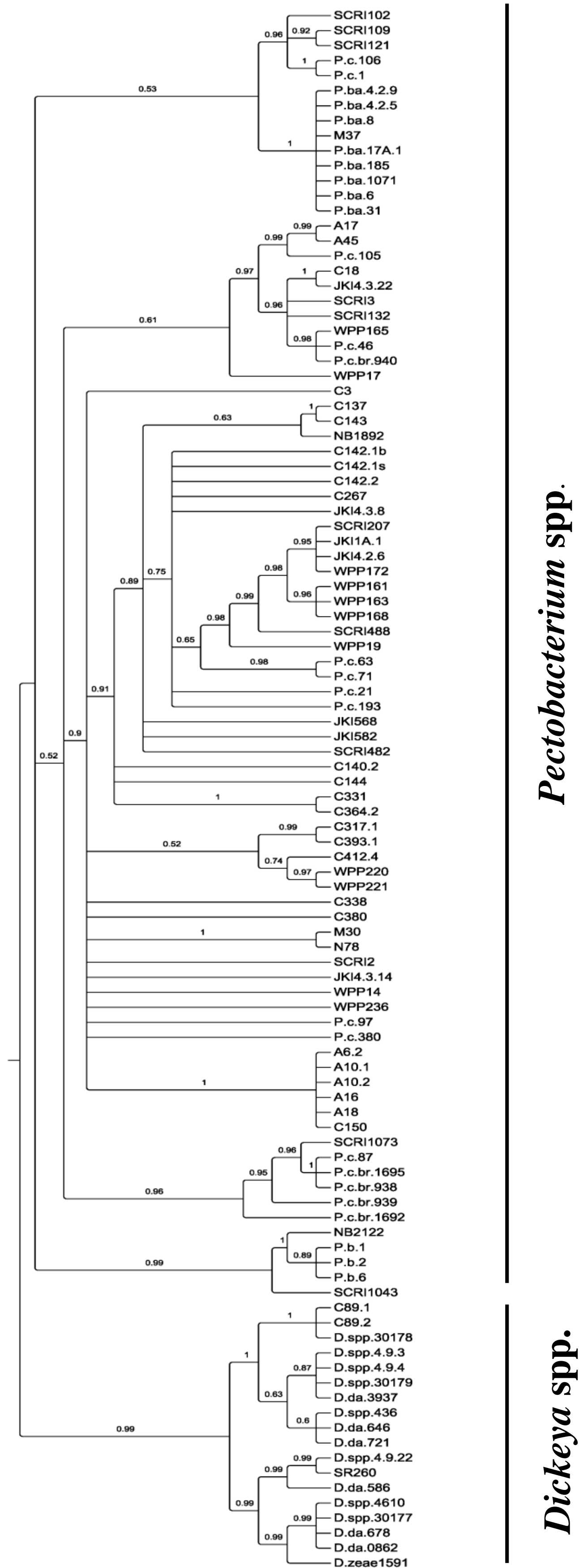


Figure S4.

ML individual tree computed for the *mtlD* gene fragments of 104 taxa.

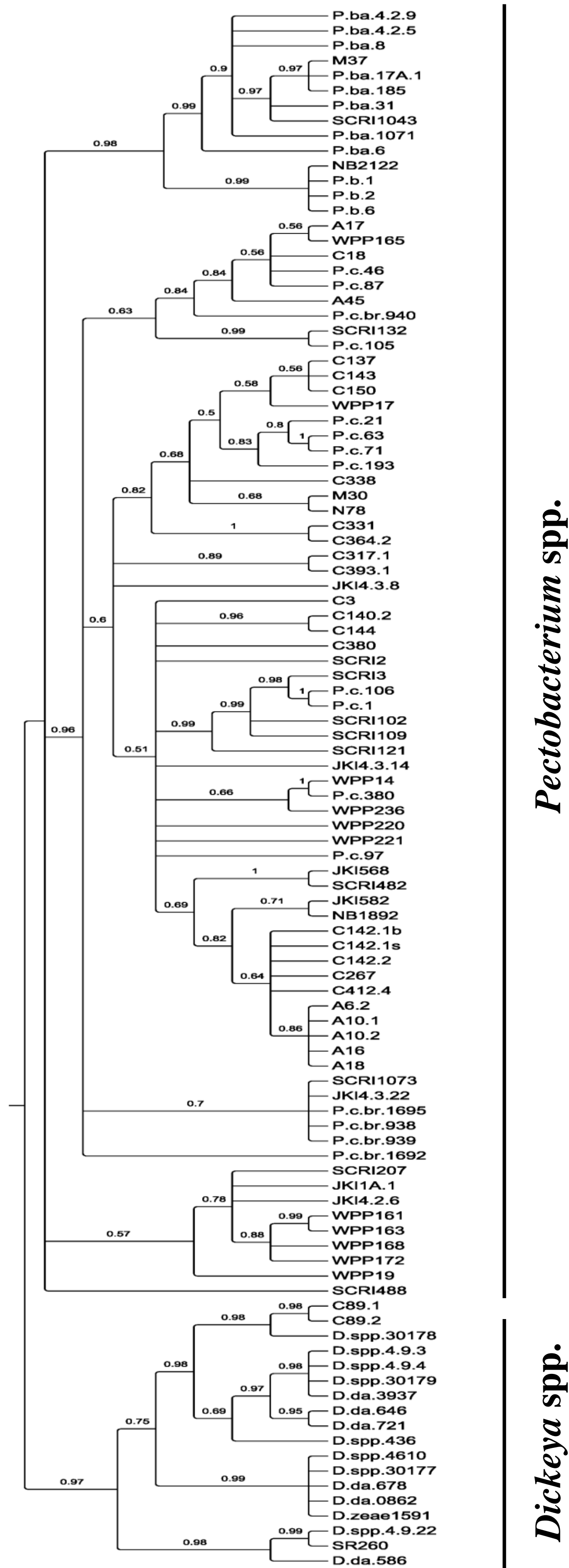


Figure S5.

ML individual tree computed for the *rpoS* gene fragments of 63 taxa.

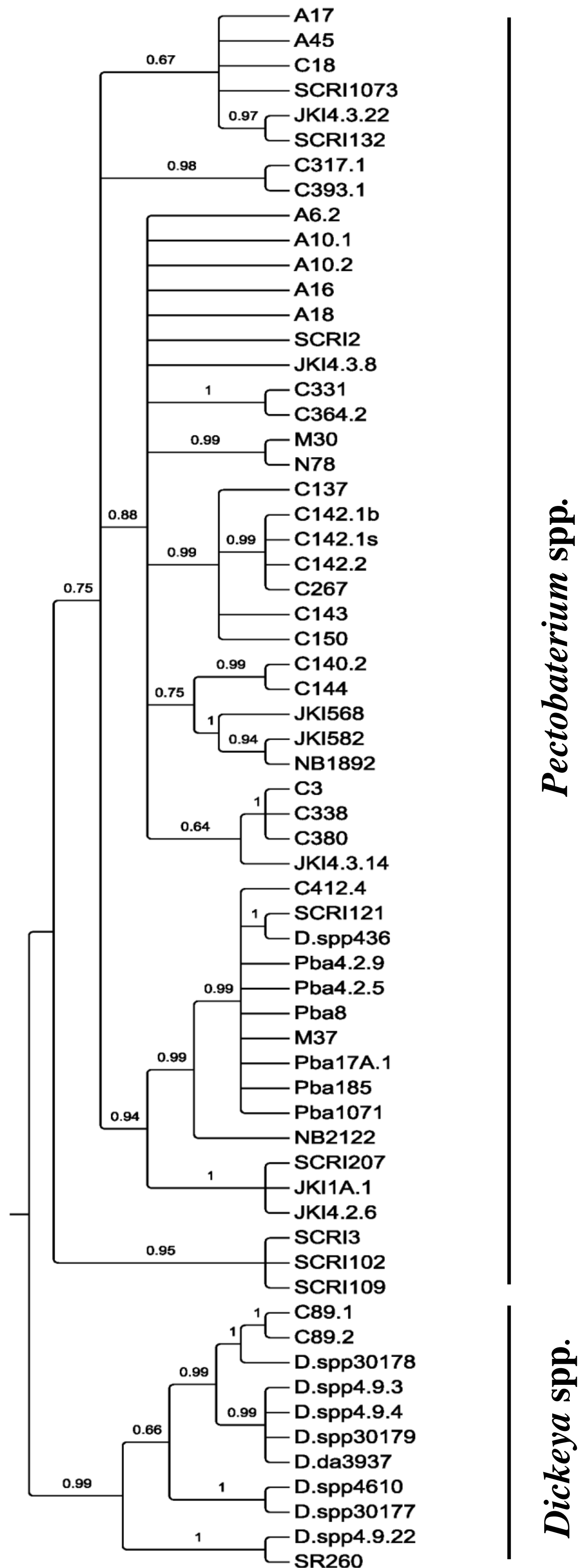


Figure S6.

ML individual tree computed for the *acnA* gene fragments of 104 taxa.

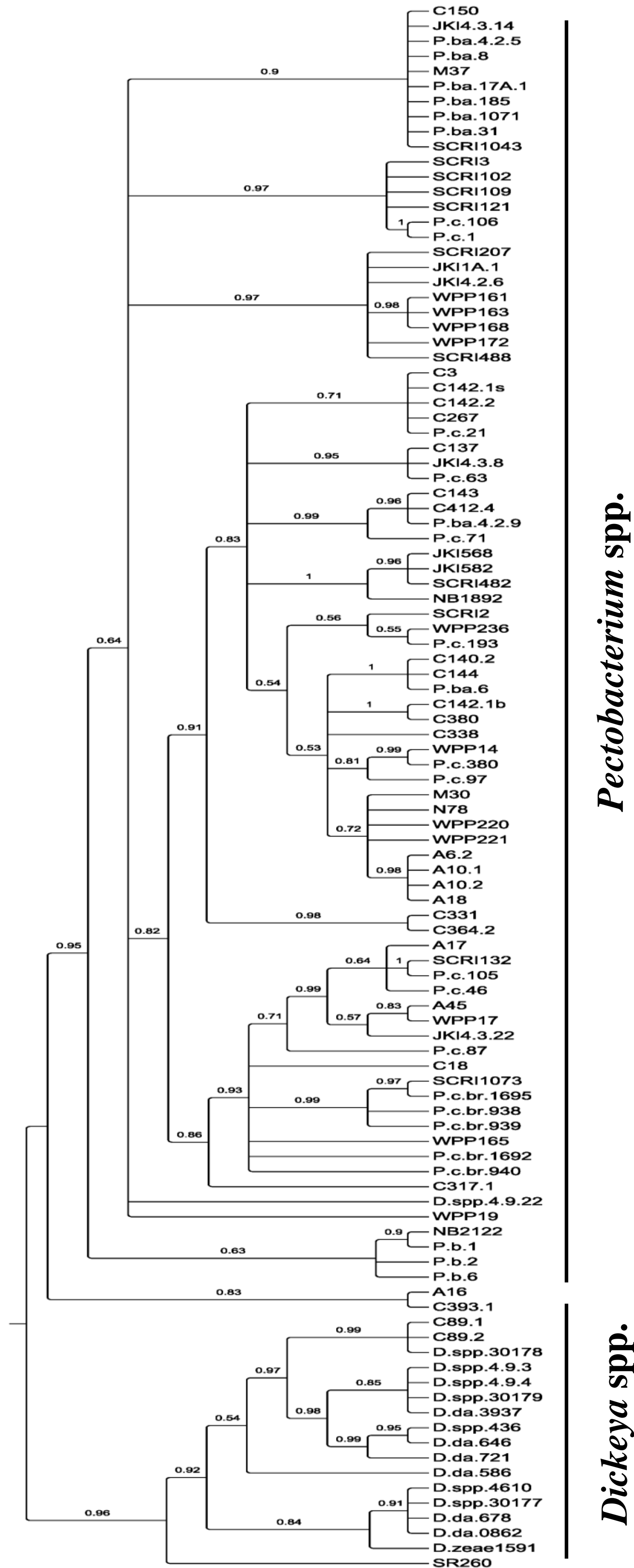


Figure S7.

ML individual tree computed for the *icdA* gene fragments of 104 taxa.

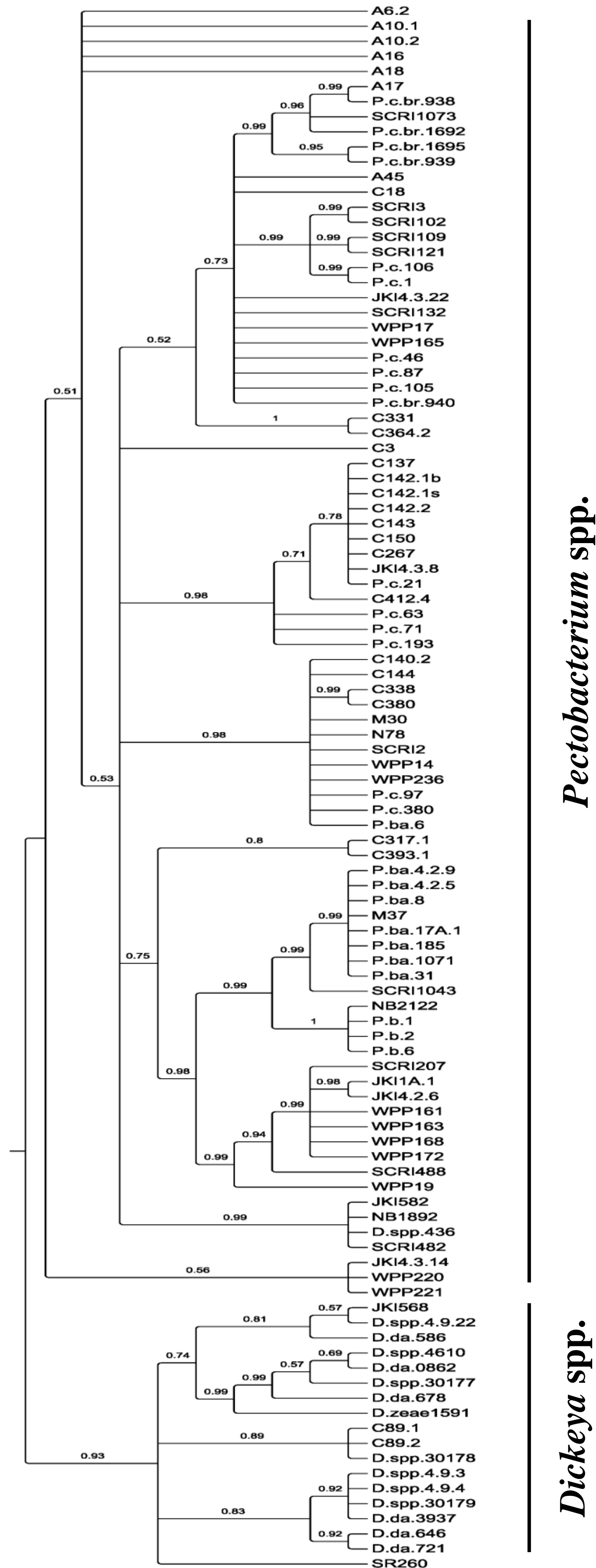


Figure S8.

ML individual tree computed for the *pgi* gene fragments of 104 taxa.

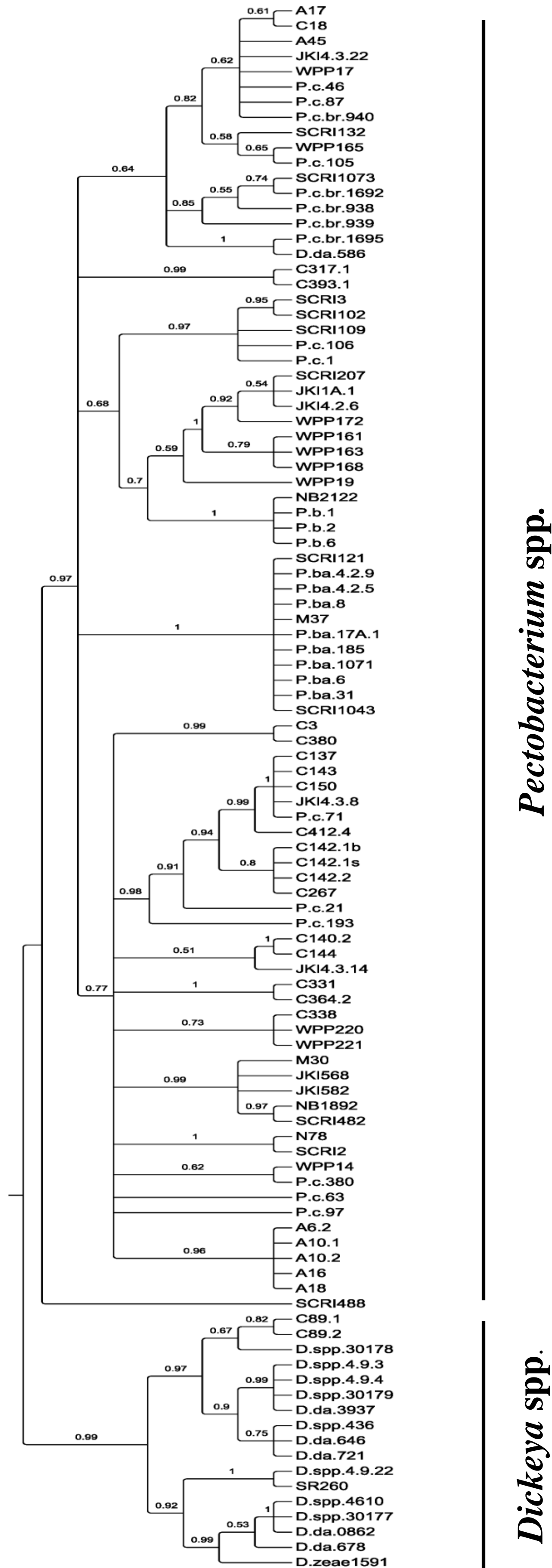


Figure S9.

ML individual tree computed for the *mdh* gene fragments of 104. taxa.

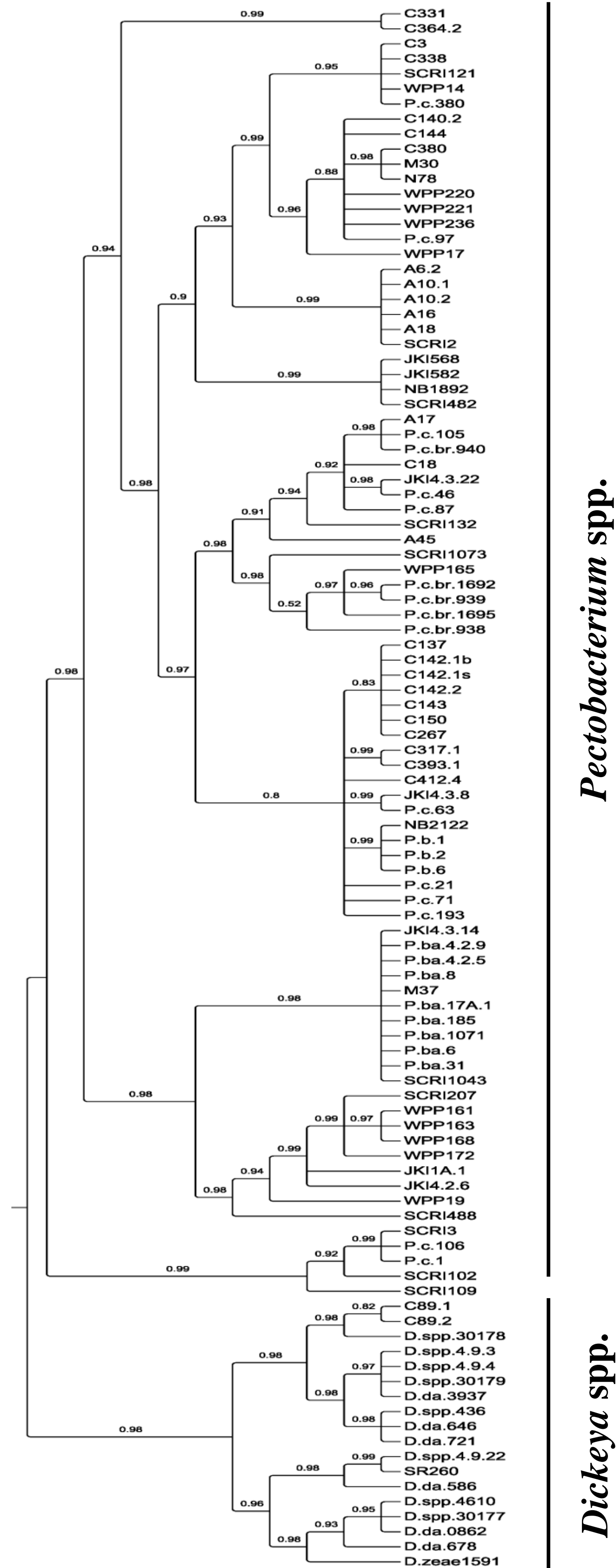


Figure S10

Dendrogram showing the evolutionary relationships among 25 strains of *P. carotovorum* subspecies based on 46 sequences representing each strain with one or two homologs (I, II) of the 16S rDNA gene determined by Maximum Likelihood method based on Hasegawa-Kishino-Yano specific model. The bootstrap consensus tree inferred from 1000 replicates. There were a total of 1530 positions in the final dataset. Bootstrapping values less than 70 % were cut off.

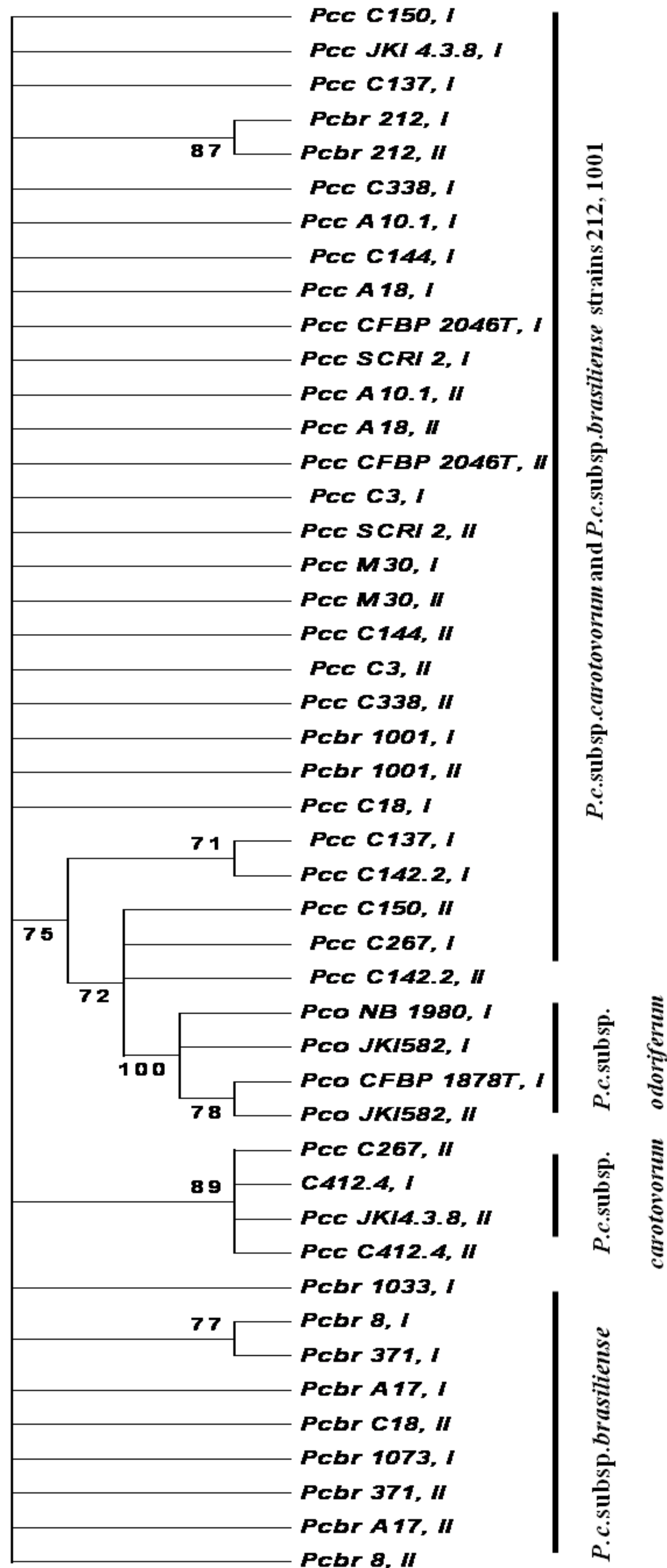


Figure S11

Dendrogram showing the evolutionary relationships among *P. carotovorum* subspecies based on 16S rDNA sequence of 25 strains determined by Maximum Likelihood method based on the Jukes-Cantor specific model. The bootstrap consensus tree inferred from 1000 replicates. There were a total of 1530 positions in the final dataset. Bootstrapping values less than 70 % were cut off.

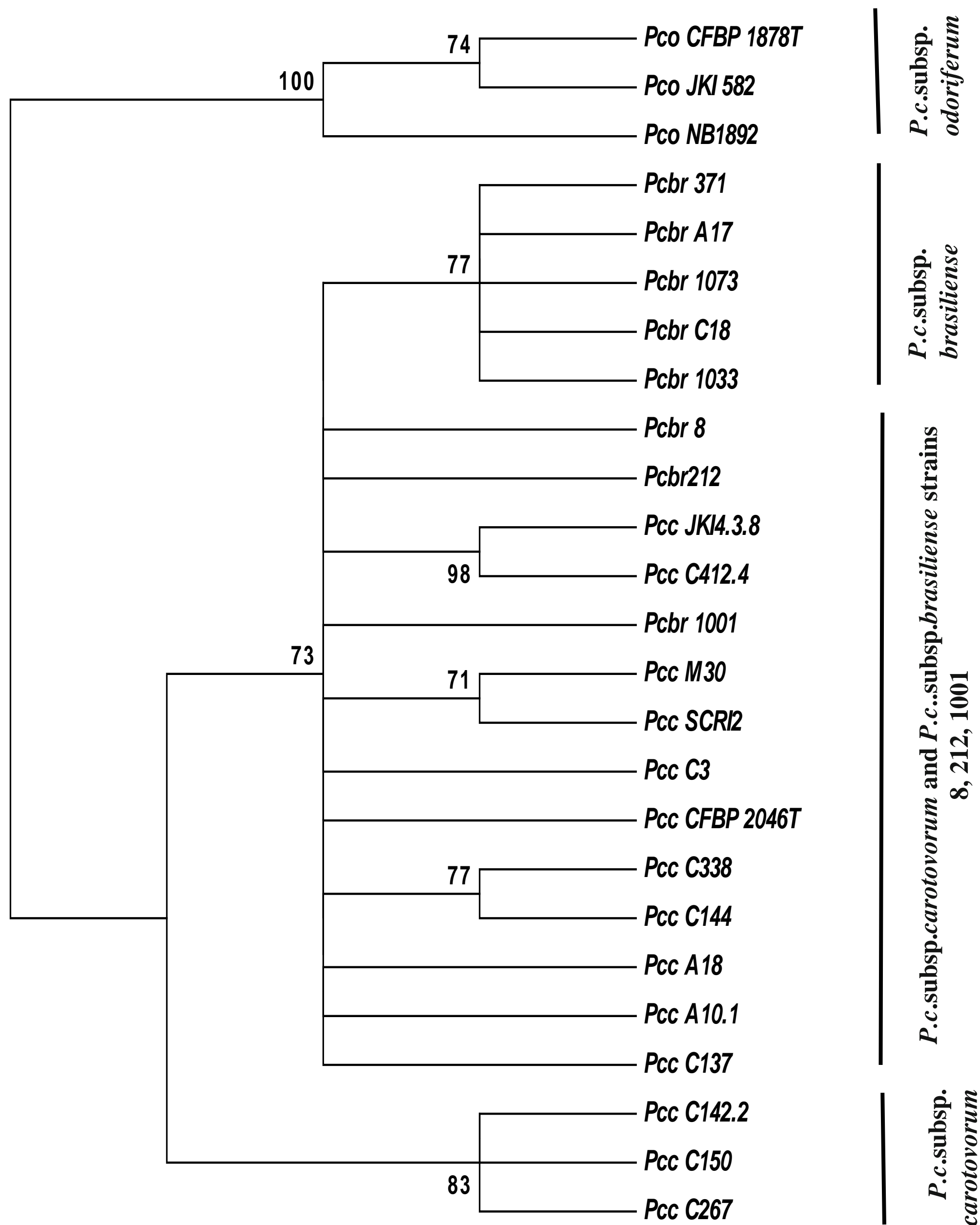


Figure S12

Dendrogram showing evolutionary relationships among *P. carotovorum* subspecies based on aminacid sequences translated from the eight gene nucleotide sequences applied to 32 strains by the Maximum Likelihood method based on the JTT specific model. The bootstrap consensus tree is inferred from 1000 replicates. Bootstrapping values less than 70 % were cut off.

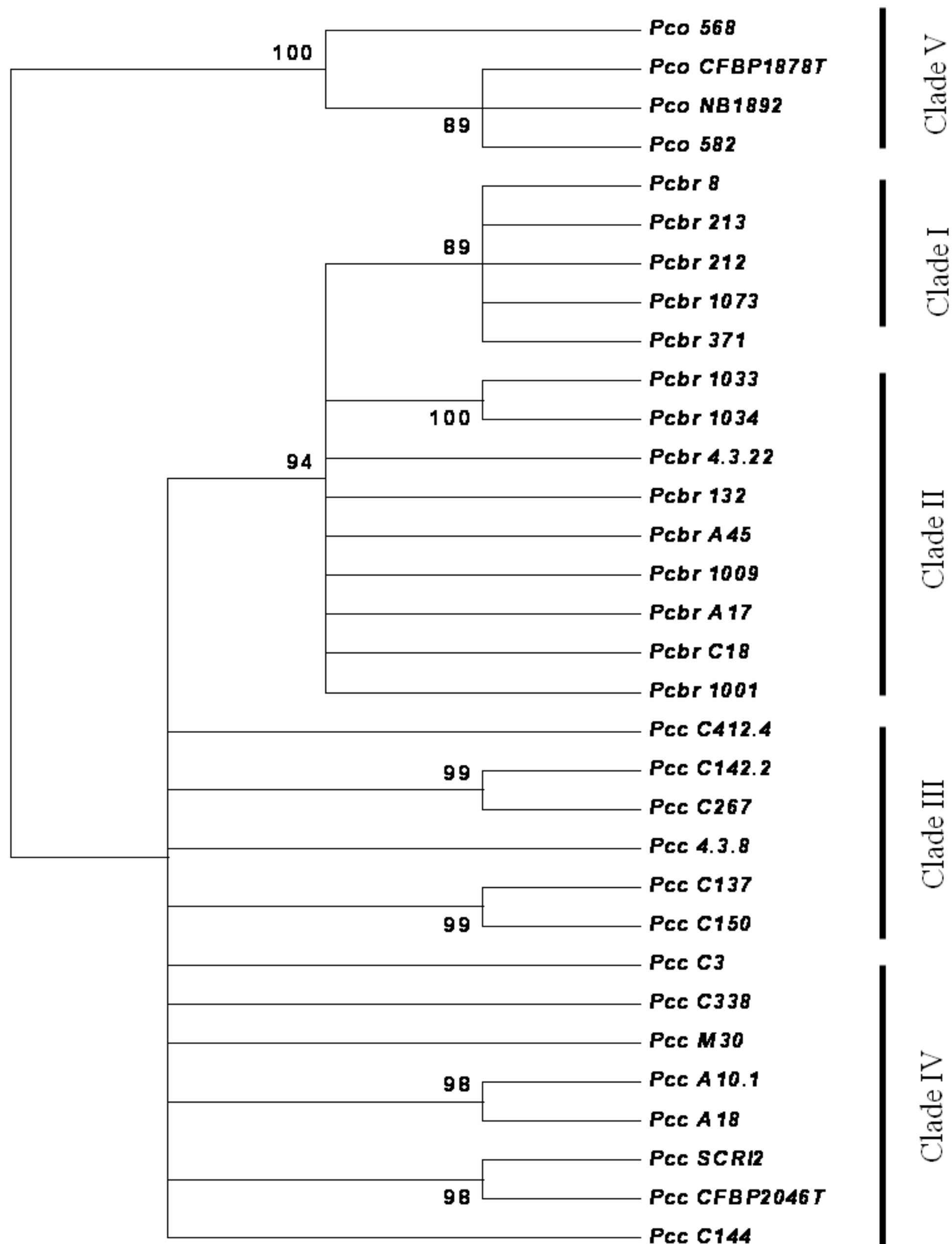


Figure S13

Dendrogram showing evolutionary relationships among the eight *Pectobacterium* taxa namely, *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum*, *P carotovorum* subsp. *brasiliense* subsp. nov., *P. atrosepticum*, *P: betavasculorum*, *P. wasabiae*, *P. aroideae* sp. nov. based on aminacid sequences translated from the eight gene nucleotide sequences applied to the eight type strains and additional 3 strains of the *P. aroideae* sp. nov. by the Maximum Likelihood method based on the JTT aminacid specific model. The bootstrap consensus tree is inferred from 1000 replicates. Bootstrapping values less than 70 % were cut off.

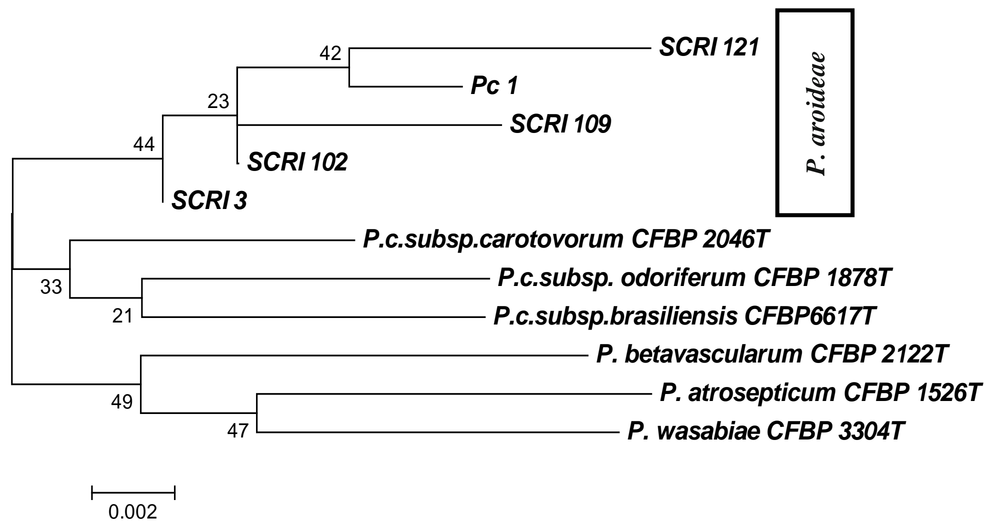
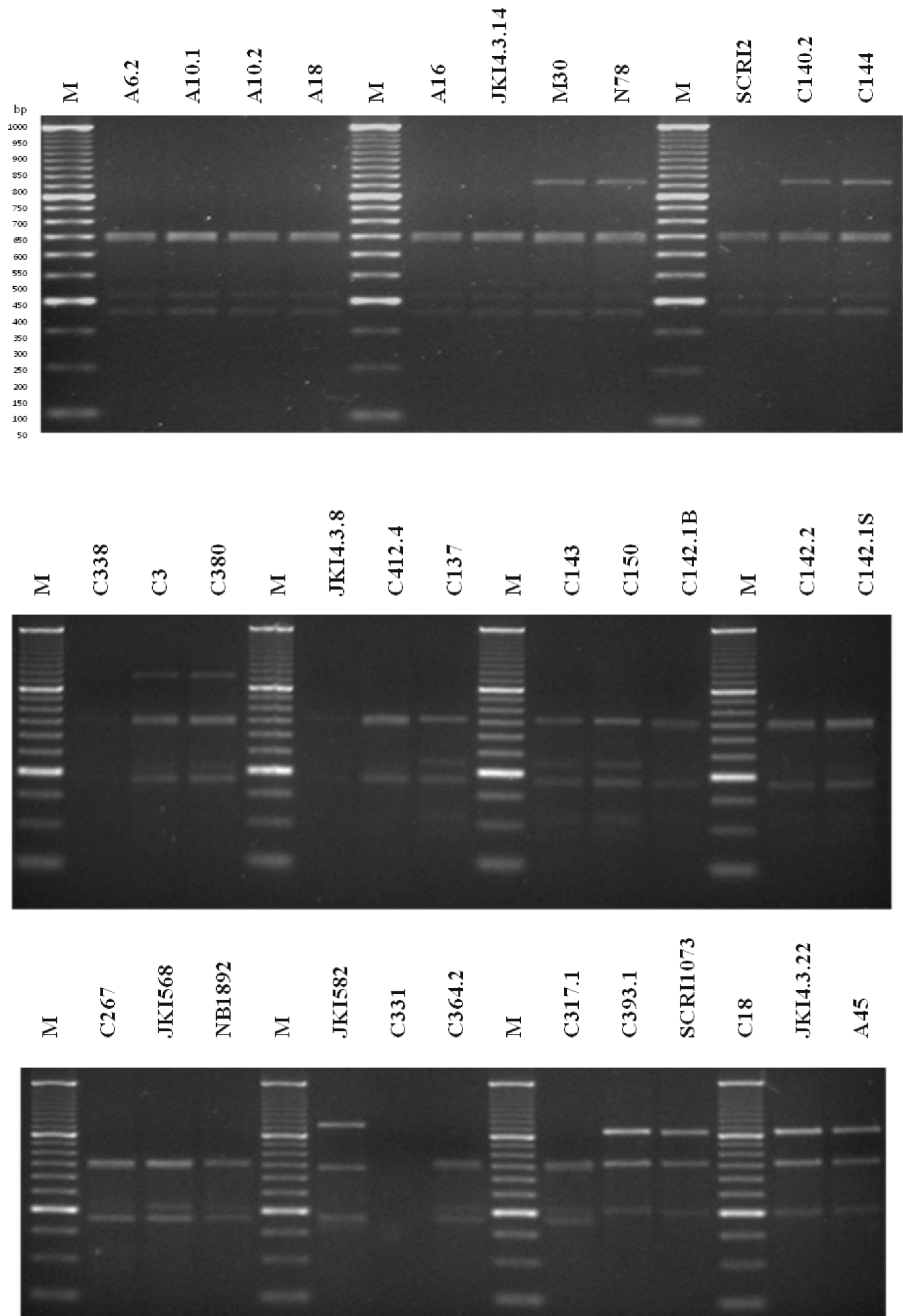
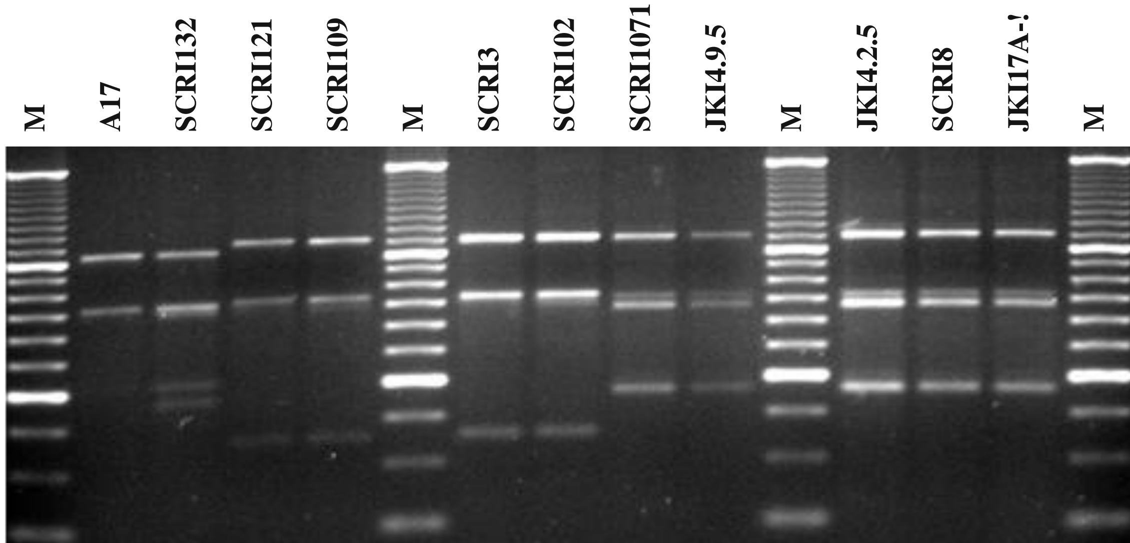
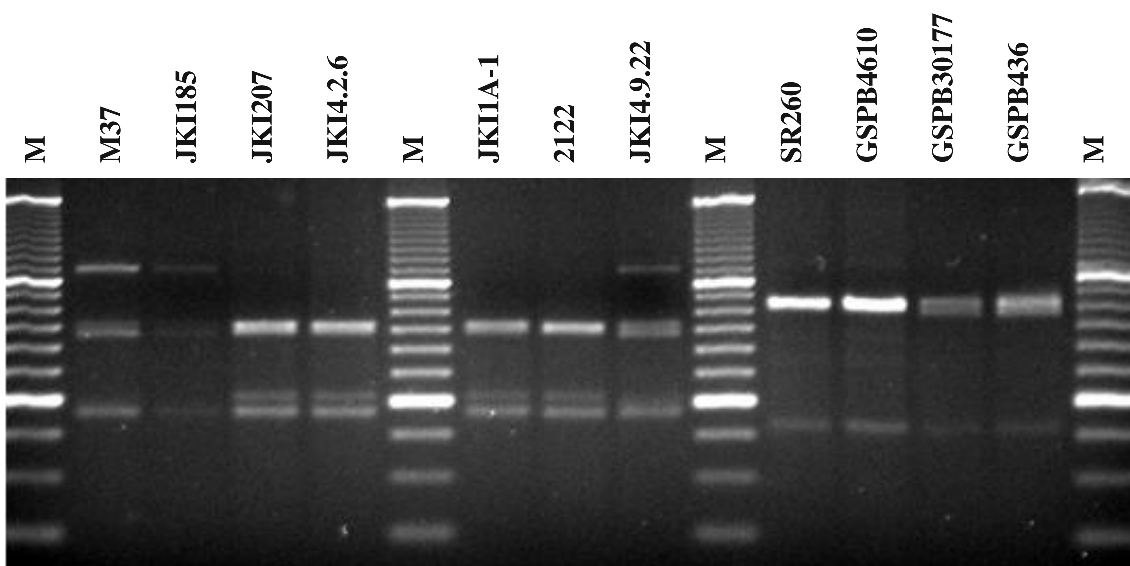


Figure S14

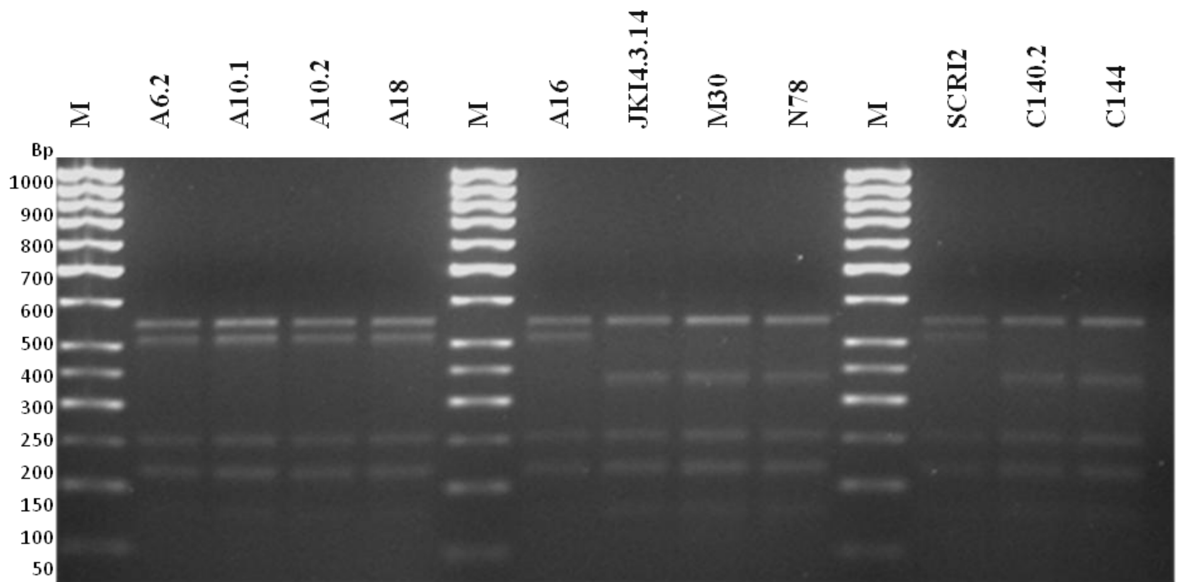
14.1. RFLP-PCR-ITS amplification patterns of *Pectobacterium* digested with *RsaI* restriction enzyme and produced variable band lengths.

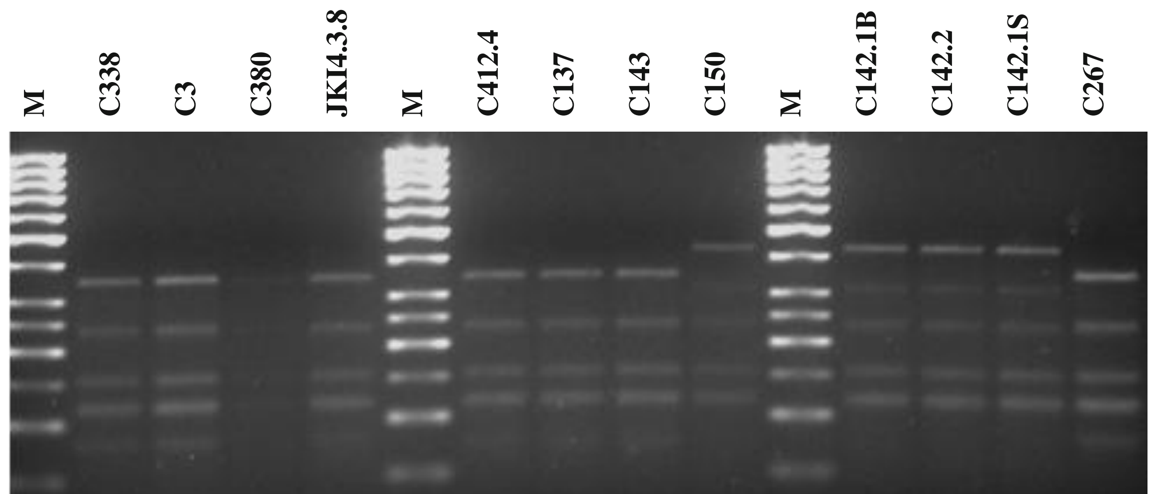


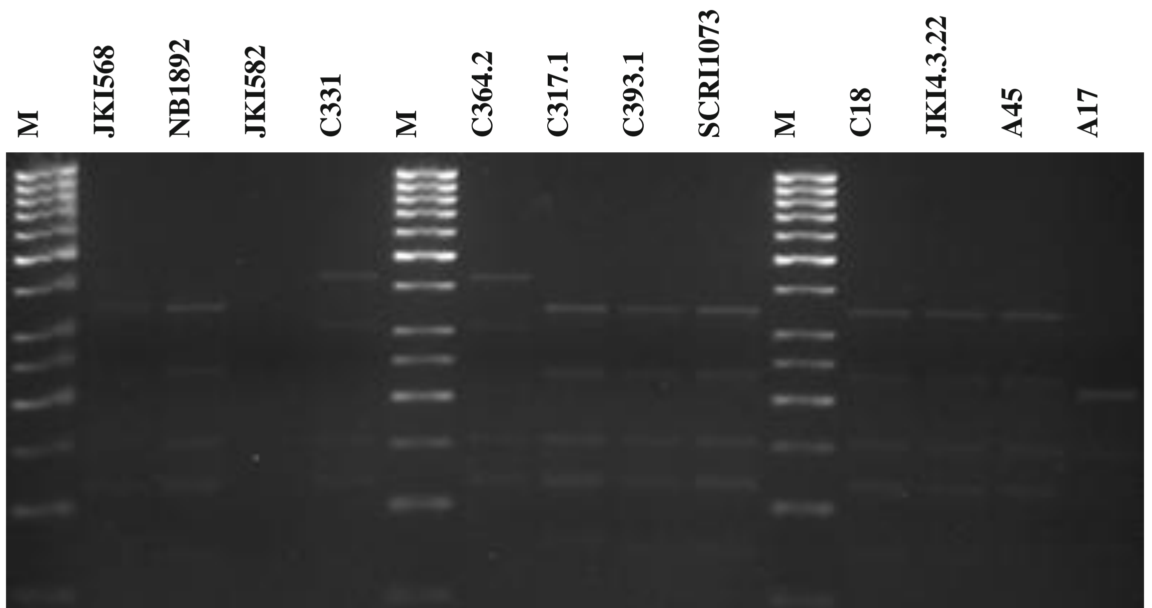

M
A17
SCRI132
SCRI121
SCRI109
M
SCRI3
SCRI102
SCRI1071
JKI4.9.5
M
JKI4.2.5
SCRI8
JKI17A-!
M

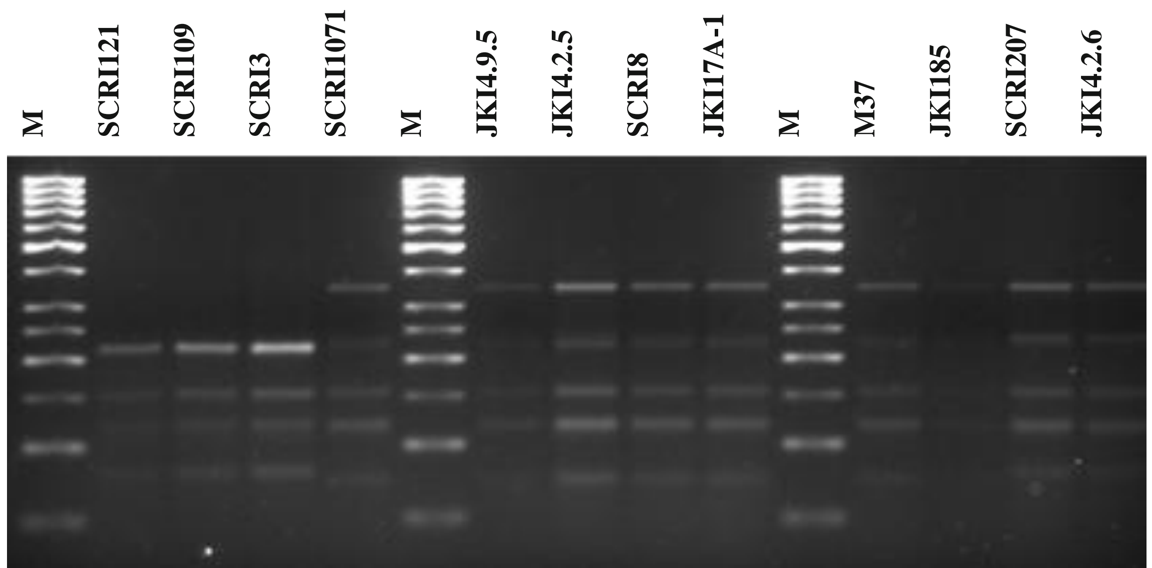

M
M37
JKI185
JKI207
JKI4.2.6
M
JKI1A-1
2122
JKI4.9.22
M
SR260
GSPB4610
GSPB30177
GSPB436
M

14.2. RFLP-PCR-ITS amplification patterns of *Pectobacterium* digested with *Hha* restriction enzyme and produced variable band lengths.








M
SCRI121
SCRI109
SCRI3
SCRI1071
M
JKI4.9.5
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SCRI8
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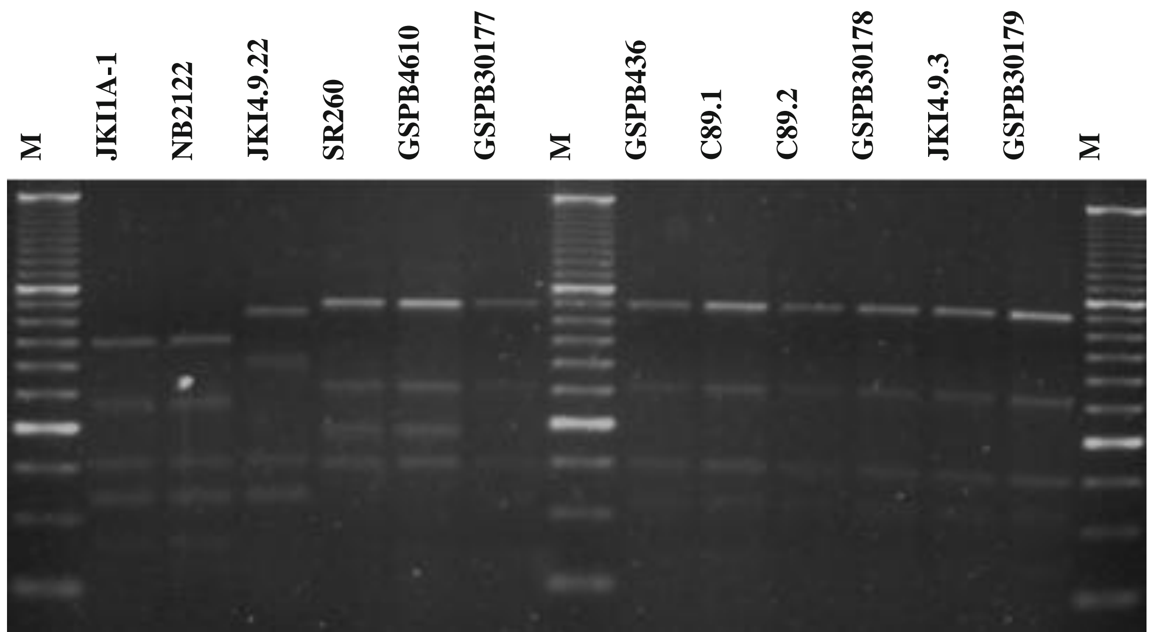
SCRI207
JKI4.2.6


M
JKI1A-1
NB2122
JKI4.9.22
SR260
GSPB4610
GSPB30177
M
GSPB436
C89.1
C89.2
GSPB30178
JKI4.9.3
GSPB30179
M

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List of publications

- The use of two complementary DNA assays, AFLP and MLSA, for epidemic and phylogenetic studies of pectolytic enterobacterial strains with focus on the heterogeneous species *P. carotovorum*

  (Plant Pathology, Doi: 10.1111/j.1365-3059.2011.02546.x)

- Taxonomic relatedness among three *Pectobacterium carotovorum* subspecies and validation of *P. carotovorum* subsp. *brasiliense* subsp. nov.

  (International Journal of Systematic and Evolutionary Microbiology, accepted)

- *Pectobacterium aroideae* sp. nov. formerly known as *Erwinia aroideae* (1904), the soft rot pathogen on monocotyledonous plant families.
  (International Journal of Systematic and Evolutionary Microbiology, to be submitted)

- Evaluation of different isolated causal agent of potato soft rot blackleg and assessment of susceptibility of ten potato cultivars under laboratory condition.
  (Arab Journal of Plant Protection, 27: 26-35, 2009)

- Survey of bacterial soft rot pathogens in potato in Syria and determination of its biochemical and Pathogenicity characteristic.
  (Arab Journal of Plant Protection, 24: 20-27, 2006)

CURRECULUM VITAE

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Nationality	Syrian
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Academic background

PhD (March, 2008-December, 2011) Taxonomic relationships among species of *Pectobacterium* and description of two novel taxa within the heterogeneous species *P. carotovorum,* namely *P. carotovorum* subsp. *brasiliense* subsp. nov. and *P. aroideae* sp. nov., Leibniz Universität Hannover, Germany.

M.Sc (2003-2006) Master of Science, plant bacteriology, Damascus University, Syria.

Diploma (2001-2002) Phytopathology, Damascus University

BSc (1994-1999) Agriculture, Plant protection, Damascus University

School Leaving Certificate Degree (1994) Hama, Syria.

Short term courses and training

- Methods in Molecular plant breeding. Summer semester, 2010.
- Practical Course on Plant Biotechnology. 02.03-20.03.2009.

Conferences and scientific activities

- 12th International Conference of Plant Pathogenic Bacteria (ICPPB). La Reunion, France, June 7-11, 2010.
  Oral presentation: Fingerprinting methods (AFLP, MLSA) for identification and characterization of pectolytic, soft rot causing bacterial strains from Syria in comparison to strains from worldwide origin.

- 12th International Conference of Plant Pathogenic Bacteria (ICPPB). La Reunion, France, June 7-11, 2010.

Poster: Metabolic diversity and molecular characterization of *Xanthomonas oryzea* pv. *oryzea* strains from West Africa.

- ‘*Erwinia’* Workshop. France, June 5-6, 2010.
  Oral presentation: Strong evidence for delineation of *Pectobacterium carotovorum* subsp. *carotovorum* into three new gen. nov. subspecies.

Languages

- English, IELTS certificate
- German, A2 certificate

DECLARATION BY CANDIDATE

I, Shaza Nabhan, hereby declare that this thesis, entitled **"Taxonomic relationships among species of *Pectobacterium* and description of two novel taxa within the heterogeneous species *P. carotovorum,* namely *P. carotovorum* subsp. *brasiliense* subsp. nov. and *P. aroideae* sp. nov."** is an original work conducted by myself and has been submitted for a degree in any other university**.**

Shaza Nabhan
Institute for Plant Disease and Plant Protection
Gottfried Wilhem Leibniz Universität Hannover, Hannover, Germany
December, 2011

Erklärung

Ich, Shaza Nabhan, erklären hiermit, dass diese dessiration mit dem Titel **"Taxonomic relationships among species of *Pectobacterium* and description of two novel taxa within the heterogeneous species *P. carotovorum,* namely *P. carotovorum* subsp. *brasiliense* subsp. nov. and *P. aroideae* sp. nov."** ist eine originelle Arbeit von mir durchgeführt und ist für einen Abschluss in keiner anderen Hochschule eingereicht.

Shaza Nabhan
Institut für Pflanzenkrankheiten und Pflanzenschutz
Gottfried Wilhem Leibniz Universität Hannover, Hannover, Deutschland
Dezember, 2011