Vol.2, No.12, 1369-1374 (2010)
doi:10.4236/ns.2010.212167

Natural Science

Assessment of a short phylogenetic marker based on comparisons of 3' end 16S rDNA and 5' end 16S-23S ITS nucleotide sequences on the genus *Xanthomonas*

Sabarimatou Yakoubou[1,2], Jean-Charles Côté[1*]

[1]Agriculture and Agri-Food Canada, Research Centre, Québec, Canada; [*]Corresponding Author: Jean-Charles.Cote@agr.gc.ca
[2]Département des Sciences Biologiques, Université du Québec à Montréal, Québec, Canada

Received 13 August 2010, revised 15 September 2010, accepted 20 September 2010.

ABSTRACT

A short phylogenetic marker previously used in the reconstruction of the Class γ-proteobacteria was assessed here at a lower taxa level, species in the genus *Xanthomonas*. This maker is 224 nucleotides in length. It is a combination of a 157 nucleotide sequence at the 3' end of the 16S rRNA gene and a 67 nucleotide sequence at the 5' end of the 16S-23S ITS sequence. A total of 23 *Xanthomonas* species were analyzed. Species from the phylogenetically related genera *Xylella* and *Stenotrophomonas* were included for comparison purposes. A bootstrapped neighbor-joining phylogenetic tree was inferred from comparative analyses of the 224 bp nucleotide sequence of all 30 bacterial strains under study. Four major Groups were revealed based on the topology of the neighbor-joining tree, Group I to IV. Group I and II contained the genera *Stenotrophomonas* and *Xylella*, respectively. Group III included five *Xanthomonas* species: *X. theicola*, *X. sacchari*, *X. albineans*, *X. transluscens* and *X. hyacinthi*. This group of *Xanthomonas* species is often referred to as the *hyacinthi* group. Group IV contained the other 18 *Xanthomonas* species. The overall topology of the neighbor-joining tree was in agreement with currently accepted phylogenetic. The short phylogenetic marker used here could resolve species from three different *Xanthomonadacea* genera: *Stenotrophomonas*, *Xylella* and *Xanthomonas*. At the level of the *Xanthomonas* genus, distant species could be distinguished, and whereas some closely-related species could be distinguished, others were undistinguishable. Pathovars could not be distinguished. We have met the resolving limit of this marker: pathovars and very closely related species from same genus.

Keywords: 16S rRNA; 16S-23S ITS; Phylogeny; *Xanthomonas*

1. INTRODUCTION

The genus *Xanthomonas* comprises 27 species. These species are primarily characterized by the production of xanthomonadins, a water-insoluble yellow pigment, and the production of an exo-polysaccharide, the xanthan gum, which is used as a thickening, stabilizing and gelling agent in food, pharmaceutics, cosmetics and oil industries [1,2]. Most *Xanthomonas* species are plant pathogens [3]. They cause diseases on several economically important plants including crucifers, Solanaceae, citrus, cotton, cereals, ornamentals, fruit and nut trees [3,4]. It is estimated that at least 124 monocotyledons and 268 dicotyledons are infected by *Xanthomonas* species [4-6].

Up to the mid-90's, the classification of *Xanthomonas* species and isolates was based on phenotypic data. The main criteria for the creation of new species rested on host specificity. The taxa "pathovar" was added to distinguish *Xanthomonas* species at the infrasubspecific level. Some species, e.g. *X. axonopodis*, *X. transluscens* or *X. campestris*, comprised more than ten, 40 and 125 pathovars, respectively [3]. Several methods were used in an attempt to classify *Xanthomonas* species and pathovars: restriction fragment-length polymorphism (RFLP) [7,8], protein profiles [9] and fatty acid methyl ester profiles [10-11]. Vauterin *et al.* reorganized the classification of the genus *Xanthomonas* based on DNA-DNA hybridization [12]. They revealed 20 DNA homology groups which they considered genomic species. Since then, other approaches based on different nucleotide sequences have been used to study the phylogeny of *Xanthomonas*: the 16S rRNA gene [13], a multilocus sequence typing (MLST), [14], the 16S-23S intergenic spacer [15], the repetitive palindromic-based

polymerase chain reaction fingerprinting (Rep-PCR) [16], the *gyrB* gene [17] and a multilocus sequence analysis (MLSA) [18]. Seven additional *Xanthomonas* species have now been described [19-25].

In a recent study [26], a short 232 bp nucleotide sequence "marker" was used to reconstruct the phylogeny of the Class γ-proteobacteria. This 232 bp marker was a combination of the last 157 bp at the 3' end of the 16S rRNA gene and the first 75 bp at the 5' end of the 16S-23S rRNA internal transcribed spacer (ITS). We showed that the 157 bp sequence was highly conserved among closely related species. Owing to its higher rate of nucleotide substitutions, the 75 bp added discriminating power among species from same genus and closely related genera from same family. This marker could reconstruct the phylogeny of the species, genera, families and Orders within the Class γ-proteobacteria in accordance with the accepted classification.

In the current study, we further assess the resolving power of this marker at a much lower taxa level: species within the genus *Xanthomonas*.

2. MATERIALS AND METHODS

2.1. Bacterial Species and Strains

A total of 25 *Xanthomonas* strains from 23 species were analyzed. Four *Xylella fastidiosa* strains and one *Stenotrophomonas maltophila* stain were added for comparison purposes. They were selected on the basis that their complete genome sequences were freely available in GenBank, at the National Center for Biotechnology Information (NCBI) completed microbial genomes database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi, August 2009). All bacterial strains and their GenBank accession numbers are listed in **Table 1**.

2.2. Sequences Analysis

First, the 16S rRNA and the 16S–23S ITS sequences of the 30 bacterial strains under study were retrieved from GenBank. Second, the 16S rRNA gene nucleotide sequences were aligned using ClustalW [27] (data not shown). The length of the nucleotide sequence most conserved was determined at 157 bp. Third, the 16S-23S ITS were aligned using ClustalW (data not shown). The length of the nucleotide sequence most conserved was determined at 67 bp. These two most conserved nucleotide sequences, the 157 bp at the 3' end of 16S, and the 67 bp at the 5' end of 16S-23S ITS, were combined into a single 224 bp sequence for each bacterial species and strain under study. This 224 bp sequence will be used here as a phylogenetic marker for the *Xanthomonas* species and related genera under study.

2.3. Phylogenetic Analyses

A neighbor-joining tree was constructed [28] based on the alignment of the 224 bp sequence of the 30 bacterial strains under study. The tree was bootstrapped using 1,000 random samples of sites from the alignment, all using CLUSTAL W software [27] at the DNA Data Bank of Japan (DDBJ) (http://clustalw.ddbj.nig.ac.jp/tope.html), with the Kimura's parameter method [29]. The neighbor-joining tree was drawn using TreeView (version 1.6.6) [30,31].

3. RESULTS AND DISCUSSION

A bootstrapped neighbor-joining tree based was inferred from the alignment of the 224 bp sequence of all 25 *Xanthomonas* species and pathovars, four *Xylella fastidiosa* strains and *Stenotrophomonas maltophila* under study (**Figure 1**). Four Groups, Group I to IV, were revealed at the 95% nucleotide sequence identities. Group I contains *Stenotrophomonas maltophila*. Group II encompasses all four *Xylella fastidiosa* strains. They share 99% nucleotide sequence identities. Group III includes five *Xanthomonas* species: *X. theicola*, *X. sacchari, X. albineans*, *X. transluscens* and *X. hyacinthi*. They share at least 96% nucleotide sequence identities. This group of *Xanthomonas* species is often referred to as the *hyacinthi* group [15]. Our results are in agreement with the first identification of the *hyacinthi* group based on the homology of their 16S rRNA [13], 16S-23S ITS [15] and *gyrB* nucleotide sequences [17] and MLSA [18]. Group IV contains 18 *Xanthomonas* species. These species share at least 95% nucleotide sequence identities. Six species can be distinguished: *X. axonopodis*, *X. codiaei*, *X. fragariae*, *X. campestris*, *X. cassavae* and *X. melonis*. *Xanthomonas perforans*, *X. euvesicatoria* and *X. alfalfae* are grouped together and appear undistinguishable. These species share 100% nucleotide sequence identities. The grouping of these six species is in agreement with the work of Parkinson *et al*. [18] based on comparison of gyrase B gene sequences. Furthermore, the grouping of *X. perforans*, *X. euvesicatoria* and *X. alfalfae* is in agreement with the work of Young *et al*. [18] based on MLSA. *Xanthomonas hortorum* and *X. vasicola*, and *X. oryzae* and *X. bromi* are grouped together, respectively, and appear undistinguishable. Both pair of species share 99% and 100% nucleotide sequence identities, respectively. Five other *Xanthomonas* species, *X. gardneri*, *X. vesicatoria*, *X. cucurbitae*, *X. arboricola* and *X. pisi* are grouped together and appear undistinguishable. These species share 100% nucleotide sequence identities. The three *X. campestris* strains appear undistiguishable. They share 100% nucleotide sequence identities. The three *X. campestris* strains appear un-

distiguishable. They share 100% nucleotide sequence identities.

Of the 23 *Xanthomonas* species under study, 15 species or group of species could be distinguished by the 224 bp sequence used as marker. Very closely related species, such as those in Group IV could not be dinstinguished. Pathovars could not be distinguished, as exemplified by the three *X. campestris* pathovars. The overall

Table 1. Bacterial species used in this study.

Genera	Species	Pathovars/Strain	GenBank accession no.
Stenotrophomonas	*maltophilia*	R551-3	NC_01107
Xanthomonas	*albilineans*	LMG 494^T	X95918
	alfalfae	F1	AF442741
	arboricola	pv. juglandis LMG 747^T	Y10757
	axonopodis	LMG 538^T	X95919
	bromi	LMG 947^T	AF209754
	campestris	pv. campestris ATCC 33913^T	NC_003902
	campestris	pv. campestris B100	NC_010688
	campestris	pv. campestris 8004	NC_007086
	cassavae	LMG 673^T	AF209756
	codiaei	LMG 8678^T	Y10765
	cucurbitae	LMG 690^T	Y10760
	euvesicatoria	85-10	NC_007508
	fragariae	LMG 708^T	X95920
	gardneri	CNPH496	AY288083
	hortorum	LMG 733^T	Y10759
	hyacinthi	LMG 739^T	Y10754
	melonis	LMG 8670^T	Y10756
	oryzae	pv. oryzae MAFF 311018	NC_007705
	perforans	CNPH411	AY288081
	pisi	LMG 847^T	Y10758
	Sacchari	LMG 471^T	Y10766
	Theicola	LMG 8684^T	Y10763
	transluscens	pv. graminis	AY247064
	vasicola	LMG 736^T	Y10755
	vesicatoria	LMG 911^T	Y10761
Xylella	*fastidiosa*	9a5c	NC_002488
		M12	NC_010513
		M23	NC_010577
		Temecula	NC_004556

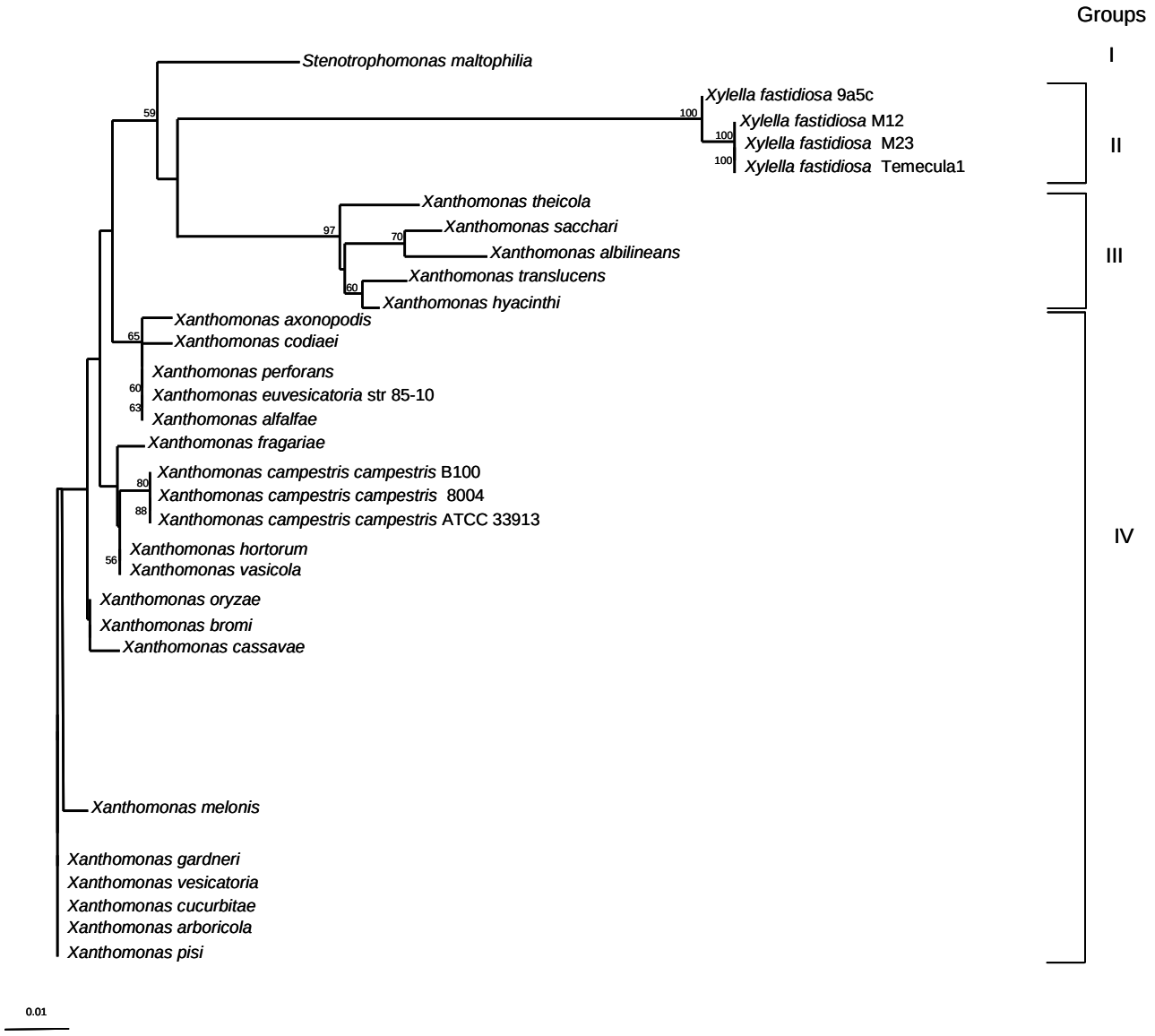

Figure 1. Bootstrapped neighbor-joining tree of the genus *Xanthomonas* species inferred from the alignment of the 224 bp marker.

topology of the neighbor-joining tree was, however, in agreement with phylogenetic trees based on the 16S rRNA [13] and the 16S-23S ITS [15].

In previous studies, we showed that a DNA sequence from 3' end 16S rRNA gene and 5' end 16S-23S ITS could be used as a marker in the reconstruction of phylogenies in the Gram-positive genus *Bacillus* and closely-related genera [32], the Gram-positive Order *Bacillales* [33], and the Gram-negative Class γ-proteobacteria [26]. This maker ranged in size from 220 bp to 232 bp. It contained 150-157 bp from the 3' end of the 16S rRNA gene and 67-75 bp from the 5' end of the 16S-23S ITS. The 150-157 bp from the 3' end of the 16S rRNA gene was often sufficient to distinguish bacterial Orders, families, and species from different genera. This sequence was, however, highly conserved among closely related species. Owing to its higher rate of nucleotide substitutions, the 67-75 bp from the 5' end of the 16S-23S ITS added resolving power among closely related species from same genus. This marker had proven useful in reconstructing the phylogenies of the genus *Bacillus* and closely-related genera [32], the Order *Bacillales* [33] and the Class γ-proteobacteria [26] in accordance with accepted phylogenies inferred from much more comprehensive datasets. This marker presented several advantages over the use of the entire 16S rRNA gene or the 16S-23S ITS or the generation of extensive phenotypic and genotypic data in phylogenetic analyses. We showed that the method was simple, rapid, suited to large screening programmes and easily accessible to most

laboratories. It also proved very valuable in revealing bacterial species which appeared misassigned and for which additional characterization appeared warranted. The resolving power of this marker has been further analyzed here in a much deeper branch of the Class γ-proteobacteria: the genus *Xanthomonas*. As expected, we have shown here that this marker could resolve species from three different *Xanthomonadacea* genera: *Stenotrophomonas*, *Xylella* and *Xanthomonas*. At the level of the *Xanthomonas* genus, distant species could be distinguished. However, although some closely-related species could be distinguished, others were grouped together and some were undistinguishable. Clearly, pathovars could not be distinguished. We have met the resolving limit of this marker: pathovars or very closely-related species.

4. CONCLUSION

A short DNA marker based on 3' end 16S rDNA and 5' end ITS, had been shown previously to be able to reconstruct the phylogeny of the Class γ-proteobacteria at the Orders, families, genera and distantly-related species levels This marker was analyzed here at a lower taxa level. First, we have shown that this marker could cluster species from same genera within the family *Xanthomonadacea*. Next, at the genus *Xanthomonas* level, we have shown that although the short DNA marker could distinguish several species, very closely-related species and pathovars could not be distinguished. We have reached the limit of the resolving power of the 224 bp sequence as a phylogenetic marker: very closely-related species and pathovars.

REFERENCES

[1] McNeely, W.H. and Kang, K.S. (1973) Xanthan and some other biosynthetic gums. In: Whistler, R.L. and BeMiller, J.N. Eds., 2nd Edition, *Industrial Gums*, Academic Press, New York, 473-497.

[2] Kennedy J.F. and Bradshaw, I.J. (1984) Production, Properties, and Applications of Xanthan. In: Bushell M. E., Ed., *Progress in Industrial Microbiology*, Elsevier, Amsterdam, 319-371.

[3] Vauterin, L., Swings, J., Kersters, K., Gillis, M., Mew, T. W., Schroth, M.N., Palleroni, N.J., Hildebrand, D.C., Stead, D.E. and other authors (1990) Towards an improved taxonomy of *Xanthomonas*. *International Journal of Systematic Bacteriology*, **40**, 312-316.

[4] Hayward, A.C. (1993) The host of *Xanthomonas*. In Swings J.G. and Civerolo E.L., Eds., *Xanthomonas*, Chapman & Hall, London, 51-54.

[5] Leyns, F., De Cleene, M., Swing, J. and De Ley, J. (1984) The host range of the genus *Xanthomonas*. *Botanical review*, **50**, 308-356.

[6] Vauterin, L., Hoste, B., Kersters, K. and Swings, J. (1995) Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology*, **45**, 472-489.

[7] Lazo, G.R., Roffey, R. and Gabriel, D.W. (1987) Pathovars of *Xanthomonas* campestris are distinguishable by restriction fragment-length polymorphism. *International Journal of Systematic Bacteriology*, **37**, 144-221.

[8] Lazo, G.R., and Gabriel, D.W. (1987) Conservation of plasmid DNA sequences and pathovar identification of strains of *Xanthomonas campestris*. *Phytopathology*, **77**, 448-453.

[9] Vauterin, L. Yang, P., Hoste, B., Vancanneyt, M., Civerolo, E.L., Swings, J. and Kersters, K. (1991) Differentiation of *Xanthomonas campestris* pv. Citri strains by sodium dodecyl; sulphate-polyacrylamide gel electrophoresis of proteins, fatty acid analysis, and DNA-DNA hybridization. *International Journal of Systematic Bacteriology*, **41**, 535-542.

[10] Chase, A.R., Stall, R.E., Hodge, N.C. and Jones, J.B. (1992) Characterization of *Xanthomonas campestris* strains from aroids using physiological, pathological, and fatty acid analyses. *Phytopathology*, **82**, 754-759.

[11] Yang, P., Vauterin, L., Vancanneyt, M., Swings, J. and Kersters, K. (1993) Application of fatty acid methyl esters for the taxonomic analysis of the genus *Xanthomonas*. *Systematic and Applied Microbiology*, **16**, 47-71.

[12] Vauterin, L., Hoste, B., Kersters, K. and Swings, J. (1995) Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology*, **45**, 472-489.

[13] Hauben, L., Vauterin, L., Swings, J. and Moore, E. (1997) Comparison of 16S ribosomal DNA sequences of all *Xanthomonas* species. *International Journal of Systematic Bacteriology*, **47**, 328-335.

[14] Maiden, M.C.J., Bygraves, J.A., Feil, E.J., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zurth, K., Caugant, D., Feavers, I.M., Achtman, M. and Spratt, B.G. (1998) Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences*, USA, 1998, **95**, 3140-3145.

[15] Gonçalves, E.R. and Rosato, Y.B. (2002) Phylogenetic analysis of *Xanthomonas* species based upon 16S–23S rDNA intergenic spacer sequences. *International Journal of Systematic and Evolutionary Microbiology*, **52**, 355-361.

[16] Rademaker, J.L.W., Louws, F.J., Schultz, M.H., Rossbach, U., Vauterin, L., Swings, J. and de Bruijn, F.J. (2005) A comprehensive species to strain taxonomic framework for *Xanthomonas*. *Phytopathology*, **95**, 1098-1111.

[17] Parkinson, N., Aritua, V., Heeney, J., Cowie, C., Bew, J. and Stead, D. (2007) Phylogenetic analysis of *Xanthomonas* species by comparison of partial gyrase B gene sequences. *International Journal of Systematic and Evolutionary Microbiology*, **57**, 2881-2887.

[18] Young, J.M., Park, D.-C., Shearman, H.M. and Fargier, E. (2008) A multilocus sequence analysis of the genus *Xanthomonas*. *Systematic and Applied Microbiology*, **31**, 366-377.

[19] Trébaol, G., Gardan, C., Manceau, J., Tanguy, Y., Trilly, Y. and Boury, S. (2000) Genomic and phenotypic characterisation of *Xanthomonas cynarae*; a new species causing bacterial bract spot of artichoke (*Cynara scolymus* L.). *International Journal of Systematic and Evolutionary Microbiology*, **50**, 1471-1478.

[20] Jones, J.B., Lacy, G.H., Bouzar, H., Stall, R.E. and Schaad, N.W. (2004) Reclassification of the xanthomonads associated with bacterial spot disease of tomato and pepper. *Systematic and Applied Microbiology*, **27**, 755-762.

[21] Euzéby, J. (2006) Validation of the publication of new names and new combinations previously effectively, but not validly published. Validation List no. 109. *International Journal of Systematic and Evolutionary Microbiology*, **56**, 925-927.

[22] Euzéby, J. (2007) List of New names and new combinations previously effectively, but not validly, published. Validation List no. 115. *International Journal of Systematic and Evolutionary Microbiology*, **57**, 893-897.

[23] Schaad, N.W., Postnikova, E., Lacy, G.H., Sechler, A., Agarkova, I., Stromberg, P.E., Stromberg, V.K. and Vidaver, A.K. (2005) Reclassification of *Xanthomonas campestris* pv. *citri* (ex Hasse 1915) Dye 1978 forms A, B/C/D, and E as *X. smithii* subsp. *citri* (ex Hasse) sp. nov. nom. rev. comb. nov., *X. fuscans* subsp. *aurantifolii* (ex Gabriel 1989) sp. nov. nom. rev. comb. nov., and *X. alfalfae* subsp. *citrumelo* (ex Riker and Jones) Gabriel et al., 1989 sp. nov. nom. rev. comb. nov.; *X. campestris* pv. malvacearum (ex Smith 1901) Dye 1978 as *X. smithii* subsp. *smithii* nov. comb. nov. nom. nov.; *X. campestris* pv. alfalfae (ex Riker and Jones, 1935) Dye 1978 as *X. alfalfae* subsp. *alfalfae* (ex Riker *et al.*, 1935) sp. nov. nom. rev.; and "var. fuscans" of *X. campestris* pv. phaseoli (ex Smith, 1987) Dye 1978 as *X. fuscans* subsp. *fuscans* sp. nov. *Systematic and Applied Microbiology*, **28**, 494-518.

[24] Schaad, N.W., Postnikova, E., Lacy, G., Sechler, A., Agarkova, I., Stromberg, P.E., Stromberg, V.K. and Vidaver, A.K. (2006) (Erratum) Emended classification of Xanthomonad pathogens on citrus. *Systematic and Applied Microbiology*, **29**, 690-695.

[25] Schaad, N.W., Postnikova, E., Lacy, G., Sechler, A., Agarkova, I., Stromberg, P. E., Stromberg, V.K. and Vidaver, A.K. (2007) *Xanthomonas alfalfae* sp. nov., *Xanthomonas* citri sp. nov. and *Xanthomonas fuscans* sp. nov. In List of new names and new combinations previously effectively, but not validly, published, Validation List no. 115. *International Journal of Systematic and Evolutionary Microbiology*, **57**, 893-897.

[26] Yakoubou, S. and Côté, J.-C., (2010) Phylogeny of γ-proteobacteria inferred from comparisons of 3' end 16S rRNA gene and 5' end 16S-23S ITS nucleotide sequences. *Natural Science*, **2**, 535-543.

[27] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673-4680.

[28] Saitou, N. and Nei, M. (1987) The neighbour-joining method: a new method of constructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406-425.

[29] Kimura, M. (1983) The neutral theory of molecular evolution. Cambridge University Press, UK.

[30] Page, R.D.M. (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Application in the Biosciences*, **12**, 357-358.

[31] Page, R.D.M. (2000) TreeView—Tree drawing software for Apple Macintosh and Windows. http://taxonomy.zoology.gla.ac.uk/rod/treeview.html.

[32] Xu, D. and Côté, J.-C. (2003) Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3' end 16S rDNA and 5' end 16S-23S ITS nucleotide sequences. *International Journal of Systematic and Evolutionary Microbiology*, **53**, 695-704.

[33] Yakoubou, S., Xu, D. and Côté, J.-C. (2010) Phylogeny of the Order *Bacillales* inferred from 3' 16S rDNA and 5' 16S-23S ITS nucleotide sequences. *Natural Science*, **2** 990-997.

