Phylogenetic structure of *Xanthomonas* determined by comparison of *gyrB* sequences

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Previously, we have produced a phylogeny of species type strains from the plant-pathogenic genus *Xanthomonas* based on *gyrB* sequences. To evaluate this locus further for species and infraspecies identification, we sequenced an additional 203 strains comprising all the pathovar reference strains (which have defined plant hosts), 67 poorly characterized pathovars, currently classified as *Xanthomonas campestris*, and 59 unidentified xanthomonads. The well-characterized pathovars grouped either in clades containing their respective species type strain or in clades containing species related to *Xanthomonas axonopodis*. The *Xanthomonas euvesicatoria*, *Xanthomonas perforans* and *Xanthomonas alfalfae* species complex, *Xanthomonas fuscans* and *Xanthomonas citri* were discriminated as *X. axonopodis*-related clades and comprised a large proportion of unidentified strains as well as 80 pathovars representing all the *X. axonopodis* pathovars and many poorly characterized pathovars, greatly increasing the plant host ranges of the constituent species. Most xanthomonads from these three large clades were isolated from a taxonomically diverse range of plant hosts, including many weed species, from field systems in India, suggesting that these lineages became established and diversified in agricultural areas in this region. The majority of these xanthomonads had minimal sequence diversity, consistent with rapid and highly extensive pathovar diversification that has occurred in relatively recent times. Low-intensity farming practices may have provided conditions conducive to pathovar development, and evidence for pathovar diversification within other regional angiosperm floras is discussed. The *gyrB* locus was sufficiently discriminating to identify diversity within many species. Seven branches or clades were sufficiently distinct to be considered as potential novel species. This study has provided a comprehensive xanthomonad classification framework and has firmly established *gyrB* sequencing as a rapid and efficient identification tool.

INTRODUCTION

Species-level xanthomonad classification has been based largely on DNA–DNA hybridization, fatty acid profiling, rep-PCR and 16S rRNA gene sequencing (Stead, 1989; Vauterin *et al.*, 1990, 1995; Rademaker *et al.*, 2005; Schaad *et al.*, 2006). However, xanthomonad identification to species level still poses significant problems for diagnostic laboratories. Consequently, it is a common but unsatisfactory practice to infer species classification by comparison of rep-PCR profiles with those of known pathovars (Saddler & Bradbury, 2005). We have established previously (Parkinson *et al.*, 2007) that *gyrB* sequences can

discriminate all the *Xanthomonas* species type strains except those of *Xanthomonas euvesicatoria*, *Xanthomonas perforans* and *Xanthomonas alfalfae*, which form a species complex with identical *gyrB* sequences and are referred to in this study as the *X. euvesicatoria* species complex. This previous study also found that the artichoke and tomato pathogens *Xanthomonas cynarae* and *Xanthomonas gardneri* grouped within the *Xanthomonas hortorum* clade. As well as the alfalfa pathogen, this previous study also included two other recently described taxa, *Xanthomonas citri* and *Xanthomonas fuscans* (Schaad *et al.*, 2005, 2006, 2007). This follow-up study will further evaluate *gyrB* sequencing as an identification tool and aims to provide a comprehensive phylogenetic framework to aid classification. By this means, the study will provide a clearer and more standard criterion for evaluation of taxa within the genus, and reduce problems that have previously been associated with *Xanthomonas* classification and taxonomy (Young *et al.*, 1991). Additionally, the study will further establish the initial *gyrB* phylogeny as a means of revealing the diversity, relatedness and evolutionary history of the genus.

Abbreviation: slc, species-level clade.

The GenBank/EMBL/DDBJ accession numbers for the *gyrB* sequences determined in this study are EU285057–EU285243, as detailed in Supplementary Table S1.

A conventional phylogenetic tree based on *gyrB* sequences and details of sequence accession numbers are available as supplementary material with the online version of this paper.

To provide a comprehensive reference set, we sequenced a further 189 strains which included all pathovar (pv.) reference strains (referred to as pathotype strains) (which have defined plant-host ranges) together with 40 unidentified (*Xanthomonas* sp.) strains. A large number of these pathovars, many of which were isolated during the 1950s and 60s, have not been fully characterized and are provisionally classified as *Xanthomonas campestris*. To differentiate these from the more extensively characterized *X. campestris* pathovars, they are subsequently referred to in this study within square brackets and indicated using asterisks in the figures. The large collection of pathovars from India (see Table 1 for names) infect a very diverse range of plant species including indigenous herbs, minor crops, trees and weeds that originate mainly from arable fields (Padhya *et al.*, 1965; Patel *et al.*, 1969; Jindal *et al.*, 1972). These pathovars are an important resource, since their classification will provide important information on xanthomonad diversity in non-crop species from a specific region, knowledge of which is currently very limited.

METHODS

Protocols used for PCR amplification of the *gyrB* locus, sequencing and phylogenetic analysis were identical to those used previously (Parkinson *et al.*, 2007) and can be found as supplementary material for this reference at IJSEM Online. The previous paper describes the phylogeny produced from a 530 nt clipped sequence from all species type strains, and provides details of the position of the sequenced region in relation to the entire *gyrB* gene. All pathovar strains used were designated reference pathotype strains, except for *X. hortorum* pv. *carotae* NCPPB 425, and were sourced from the NCPPB (National Collection of Plant Pathogenic Bacteria, Central Science Laboratory, Sand Hutton, York, UK) unless indicated. The following strains were from other collections: [*X. campestris*] pv. *thespesiae* LMG 9057, [*X. campestris*] pv. *centellae* LMG 3245, *Xanthomonas arboricola* pv. *fragariae* LMG 19145 were from the LMG Collection (Laboratorium voor Microbiologie en Microbiele Genetica, Rijksuniversiteit, Gent, Belgium) and *X. citri* subsp. *malvacearum* ICMP 217^T and [*X. campestris*] pv. *brunneivaginae* ICMP 9991 were from the ICMP International Collection (Landcare Research, Auckland, New Zealand). Unidentified xanthomonad isolates (*Xanthomonas* sp.) were from the NCPPB collection and are listed in Table 1, which also lists the country of origin and host plant. Plant-host range and susceptibility studies for many pathovars have been reviewed by Bradbury (1986). References supporting pathovar status are listed in the bacterial names section of the International Society for Plant Pathogenic Bacteria website (<http://www.isppweb.org>). The circular phylogeny was produced using the online Interactive Tree of Life resource (<http://itol.embl.de/>; Letunic & Bork, 2007). To ensure accuracy, all sequences were verified by comparison of both forward and reverse sequences, nearly all of which were determined from independently produced subcultures. Strain 7830 used to root the phylogenies shares 82% sequence identity to *Stenotrophomonas maltophilia* ICMP 17033^T (GenBank accession no. EU499066.1) and 54% to the closest xanthomonad sequence. This latter sequence can also be used to root the phylogenies.

RESULTS

GenBank accession numbers for the *gyrB* sequences are provided in Supplementary Table S1, available in IJSEM

Online. In agreement with other studies, we found that the pathotype strains of [*X. campestris*] pv. *tardicrescens* and *X. hortorum* pv. *carotae* were not xanthomonads. Limited 16S rRNA gene sequencing indicated that *X. campestris* pv. *brunneivaginae* and an unidentified xanthomonad also from rice (NCPPB 2925) were probably *Sphingomonas* strains. The non-pigmented *Pseudomonas cissicola* type strain, which has been reported as a member of *Xanthomonas* (Hu *et al.*, 1997), was placed in the *X. citri* subsp. *citri* clade. Other unidentified presumptive xanthomonads NCPPB strains 1683, 1773, 1774, 1775, 1829, 1981, 2147, 2497, 2632, 2925 and 4210 were found by 16S rRNA gene sequencing not to belong to the genus.

The circular phylogeny (Fig. 1) provides an overview of xanthomonad relatedness determined from *gyrB* sequence comparisons. A conventional phylogeny is provided as Supplementary Fig. S1. For both phylogenies, trinomials separated by full stops are the species and pathovar designations. The composition of clades which are identified according to known species type strains or as novel species-level clades is provided in Table 1. The largest clade, furthest from the *Stenotrophomonas* root, contains the *X. euvesicatoria* species complex and comprised 63 strains, all with minimal *gyrB* sequence diversity. Two further clades, respectively containing the newly described species *X. citri* and *X. fuscans*, comprised 21 [*X. campestris*] pathovars, 16 *Xanthomonas axonopodis* pathovars and five unidentified isolates. These three species are all related to *X. axonopodis*, and many of the pathovars were isolated from a taxonomically wide range of plants, many of which occur as agricultural weeds, in India. A sister clade to that containing *X. euvesicatoria* was identified which contained eight xanthomonads including three *X. axonopodis* pathovars of bean, begonia and manihot (pathovars *phaseoli*, *begoniae* and *manihotis*, respectively). Further clades within this group distinguished the type strain of *X. axonopodis* (along with the sugar-cane pathogen *X. axonopodis* pv. *vasculorum*) from *Xanthomonas melonis*, which grouped with [*X. campestris*] pv. *lantanae*. Pathovars within *X. campestris*, *Xanthomonas translucens*, *Xanthomonas oryzae* and *X. arboricola* grouped within clades containing their respective type strain. Other *Xanthomonas* sp. and [*X. campestris*] strains that were assigned to clades that increased the host range of existing species (including eight *Xanthomonas* sp. isolates assigned to *Xanthomonas sacchari*) are referred to in the discussion. The host ranges for *Xanthomonas albilineans*, *X. bromi*, *X. cucurbitae*, *X. fragariae*, *X. hyacinthi*, *X. pisi*, *X. populi*, *X. theicola* and *X. vesicatoria* were not increased.

The locus identified infraspecies diversity in many species, and most of the pathovars belonging to *X. arboricola*, *X. translucens*, *X. hortorum* and *X. campestris* were discriminated. Strains with distinct *gyrB* sequences and correspondingly long branch lengths relative to other recognized species (Fig. 2) are identified as species-level clades (slc) 1–7. Three [*X. campestris*] pathovars constitute slc 1 and are pathogens of *Zinnia elegans*, *Hibiscus esculentus* and

Table 1. Strains used in this study and assignment to *gyrB* clades

Clades discussed in the text appear as bold headings. Slc, Species-level clade. [*X. campestris*] indicates poorly characterized *X. campestris* pathovars. All species and subspecies type strains have been sequenced previously (Parkinson *et al.*, 2007). The following type strains used in this study occur as distinct clades: *X. albilineans* NCPPB 2969^T, *X. bromi* NCPPB 4343^T, *X. cassavae* NCPPB 101^T, *X. fragariae* NCPPB 1469^T, *X. hyacinthi* NCPPB 599^T, *X. pisi* NCPPB 762^T, *X. theicola* NCPPB 4353^T and *X. vesicatoria* NCPPB 422^T. Others are listed below their respective clades.

Strain	Host	Country
<i>X. euvesicatoria</i> species complex		
<i>X. euvesicatoria</i> NCPPB 2968 ^T	<i>Capsicum frutescens</i>	USA
<i>X. perforans</i> NCPPB 4321 ^T	<i>Lycopersicon esculentum</i>	USA
<i>X. alfalfae</i> subsp. <i>alfalfae</i> LMG 495 ^T	<i>Medicago sativa</i>	India
<i>X. axonopodis</i> pv. <i>biophyti</i> NCPPB 2228	<i>Biophytum sensitivum</i>	India
<i>X. axonopodis</i> pv. <i>alfalfae</i> NCPPB 2062	<i>Medicago sativa</i>	Sudan
<i>X. alfalfae</i> subsp. <i>citrumelonis</i> LMG 9325 ^T	<i>Citrus</i> sp.	USA
<i>X. axonopodis</i> pv. <i>cyamopsidis</i> NCPPB 637	<i>Cyamopsis tetragonolobus</i>	India
<i>X. axonopodis</i> pv. <i>betlicola</i> NCPPB 2972	<i>Piper betle</i>	India
<i>X. axonopodis</i> pv. <i>cassiae</i> NCPPB 2973	<i>Cassia tora</i>	India
<i>X. axonopodis</i> pv. <i>coracanae</i> NCPPB 1786	<i>Eleusine coracana</i>	India
<i>X. axonopodis</i> pv. <i>desmodii</i> NCPPB 481	<i>Desmodium diffusum</i>	India
<i>X. axonopodis</i> pv. <i>desmodiiganeticum</i> NCPPB 577	<i>Desmodium gangeticum</i>	India
<i>X. axonopodis</i> pv. <i>desmodirotundifolii</i> NCPPB 885	<i>Desmodium rotundifolium</i>	India
<i>X. axonopodis</i> pv. <i>erythrinae</i> NCPPB 578	<i>Erythrina indica</i>	India
<i>X. axonopodis</i> pv. <i>fascicularis</i> NCPPB 2230	<i>Corchorus fascicularis</i>	India
<i>X. axonopodis</i> pv. <i>lespedezae</i> NCPPB 993	<i>Lespedeza</i> sp.	USA
<i>X. axonopodis</i> pv. <i>maculifoliigardeniae</i> NCPPB 971	<i>Gardenia</i> sp.	USA
<i>X. axonopodis</i> pv. <i>nakataecorchorii</i> NCPPB 1337	<i>Corchorus acutangulus</i>	India
<i>X. axonopodis</i> pv. <i>patelii</i> NCPPB 840	<i>Crotalaria juncea</i>	India
<i>X. axonopodis</i> pv. <i>pedalii</i> NCPPB 2368	<i>Pedaliium mure</i>	India
<i>X. axonopodis</i> pv. <i>phyllanthi</i> NCPPB 2066	<i>Phyllanthus niruri</i>	Sudan
<i>X. axonopodis</i> pv. <i>physalidicola</i> NCPPB 761	<i>Physalis alkekengi</i>	Japan
<i>X. axonopodis</i> pv. <i>poinsetticola</i> NCPPB 581	<i>Euphorbia pulcherrima</i>	India
<i>X. axonopodis</i> pv. <i>ricini</i> NCPPB 1063	<i>Ricinus communis</i>	Ethiopia
<i>X. axonopodis</i> pv. <i>tamarindi</i> NCPPB 584	<i>Tamarindus indica</i>	India
[<i>X. campestris</i>] pv. <i>alangii</i> NCPPB 1336	<i>Alangium lamarckii</i>	India
[<i>X. campestris</i>] pv. <i>amaranthicola</i> NCPPB 570	<i>Amaranthus viridis</i>	India
[<i>X. campestris</i>] pv. <i>amorphophalli</i> NCPPB 2371	<i>Amorphophallus campanulatus</i>	India
[<i>X. campestris</i>] pv. <i>argemones</i> NCPPB 1593	<i>Argemone mexicana</i>	India
[<i>X. campestris</i>] pv. <i>betae</i> NCPPB 2592	<i>Beta vulgaris</i>	Brazil
[<i>X. campestris</i>] pv. <i>blepharidis</i> NCPPB 1757	<i>Blepharis boarhaavifolia</i>	India
[<i>X. campestris</i>] pv. <i>carissae</i> NCPPB 2373	<i>Carissa carandas</i>	India
[<i>X. campestris</i>] pv. <i>clerodendri</i> NCPPB 575	<i>Clerodendron</i> sp.	India
[<i>X. campestris</i>] pv. <i>convolvuli</i> NCPPB 2498	<i>Convolvulus arvensis</i>	India
[<i>X. campestris</i>] pv. <i>coriandri</i> NCPPB 1758	<i>Coriandrum sativum</i>	India
[<i>X. campestris</i>] pv. <i>daturae</i> NCPPB 2932	<i>Datura metel</i>	India
[<i>X. campestris</i>] pv. <i>euphorbiae</i> NCPPB 1828	<i>Euphorbia acalyphoides</i>	Sudan
[<i>X. campestris</i>] pv. <i>fici</i> NCPPB 2372	<i>Ficus religiosa</i>	India
[<i>X. campestris</i>] pv. <i>heliotropii</i> NCPPB 2057	<i>Heliotropium sudanicum</i>	Sudan
[<i>X. campestris</i>] pv. <i>ionidii</i> NCPPB 1334	<i>Ionidium heterophyllum</i>	India
[<i>X. campestris</i>] pv. <i>lawsoniae</i> NCPPB 579	<i>Lawsonia inermis</i>	India
[<i>X. campestris</i>] pv. <i>mirabilis</i> NCPPB 4348	<i>Mirabilis jalapa</i>	India
[<i>X. campestris</i>] pv. <i>olitorii</i> NCPPB 464	<i>Corchorus olitorius</i>	Sudan
[<i>X. campestris</i>] pv. <i>paullinae</i> NCPPB 3079	<i>Paullinia cupana</i>	Brazil
[<i>X. campestris</i>] pv. <i>pennamericanum</i> NCPPB 4349	<i>Pennisetum americanum</i>	Nigeria
[<i>X. campestris</i>] pv. <i>physalidis</i> NCPPB 1756	<i>Physalis minima</i>	India
[<i>X. campestris</i>] pv. <i>sesami</i> NCPPB 631	<i>Sesamum indicum</i>	Sudan
[<i>X. campestris</i>] pv. <i>spermacoces</i> NCPPB 1760	<i>Spermacoce hispida</i>	India
[<i>X. campestris</i>] pv. <i>tribuli</i> NCPPB 1454	<i>Tribulus terrestris</i>	India
[<i>X. campestris</i>] pv. <i>uppalii</i> NCPPB 586	<i>Ipomoea muricata</i>	India
[<i>X. campestris</i>] pv. <i>vernoniae</i> NCPPB 1787	<i>Vernonia cinerea</i>	India

Table 1. cont.

Strain	Host	Country
[<i>X. campestris</i>] pv. <i>viegasii</i> NCPPB 4351	<i>Pachystachys lutea</i>	Brazil
[<i>X. campestris</i>] pv. <i>zingibericola</i> NCPPB 4352	<i>Zingiber mioga</i>	China
<i>Xanthomonas</i> sp. NCPPB 1062	<i>Dioscorea esculenta</i>	Papua New Guinea
<i>Xanthomonas</i> sp. NCPPB 1754	<i>Celsia coramandeliana</i>	India
<i>Xanthomonas</i> sp. NCPPB 3583	<i>Strelitzia reginae</i>	USA
<i>Xanthomonas</i> sp. NCPPB 3942	<i>Allium cepa</i>	Barbados
<i>Xanthomonas</i> sp. NCPPB 3943	<i>Allium cepa</i>	Barbados
<i>Xanthomonas</i> sp. NCPPB 3944	<i>Allium cepa</i>	Barbados
<i>X. euvesicatoria</i> species complex sister clade		
<i>X. axonopodis</i> pv. <i>manihotis</i> NCPPB 1834	<i>Manihot esculenta</i>	Brazil
<i>X. axonopodis</i> pv. <i>dieffenbachiae</i> NCPPB 1833	<i>Anthurium</i> sp.	Brazil
<i>X. axonopodis</i> pv. <i>begoniae</i> NCPPB 1926	<i>Begonia tuberhybrida</i>	New Zealand
<i>X. axonopodis</i> pv. <i>phaseoli</i> NCPPB 3035	<i>Phaseolus vulgaris</i>	USA
[<i>X. campestris</i>] pv. <i>passiflorae</i> NCPPB 2346	<i>Passiflora edulis</i>	Brazil
[<i>X. campestris</i>] pv. <i>syngonii</i> NCPPB 3586	<i>Syngonium podophyllum</i>	USA
<i>X. axonopodis</i> pv. <i>begoniae</i> NCPPB 3003	<i>Begonia</i> sp.	New Zealand
<i>Xanthomonas</i> sp. NCPPB 3569	<i>Ipomoea batatas</i>	Venezuela
<i>Xanthomonas</i> sp. NCPPB 3761	<i>Pisum sativum</i>	India
<i>X. citri</i> subsp. <i>citri</i>		
<i>X. citri</i> subsp. <i>citri</i> LMG 9322 ^T	<i>Citrus</i> sp.	USA
[<i>X. campestris</i>] pv. <i>vitistrifoliae</i> NCPPB 1451	<i>Vitis trifolia</i>	India
[<i>X. campestris</i>] pv. <i>viticola</i> NCPPB 2475	<i>Vitis vinifera</i>	India
<i>Xanthomonas</i> sp. NCPPB 2934	<i>Cayratia carnosa</i>	India
<i>X. axonopodis</i> pv. <i>khayae</i> NCPPB 536	<i>Khaya senegalensis</i>	Sudan
<i>X. axonopodis</i> pv. <i>martyniicola</i> NCPPB 1148	<i>Martynia diandra</i>	India
[<i>X. campestris</i>] pv. <i>vitiscarnosae</i> NCPPB 1149	<i>Vitis carnosa</i>	India
[<i>X. campestris</i>] pv. <i>vitiswoodrowii</i> NCPPB 1014	<i>Vitis woodrowii</i>	India
<i>Xanthomonas</i> sp. (<i>Pseudomonas cissicola</i>) NCPPB 2982	<i>Cissus japonica</i>	Japan
[<i>X. campestris</i>] pv. <i>mangiferaeindicae</i> NCPPB 490	<i>Mangifera indica</i>	India
[<i>X. campestris</i>] pv. <i>centellae</i> NCPPB 3245	<i>Centella asiatica</i>	India
[<i>X. campestris</i>] pv. <i>durantae</i> NCPPB 1456	<i>Duranta repens</i>	India
<i>X. axonopodis</i> pv. <i>clitoriae</i> NCPPB 3092	<i>Clitoria biflora</i>	India
<i>X. axonopodis</i> pv. <i>citri</i> NCPPB 409	<i>Citrus limon</i>	New Zealand
<i>X. axonopodis</i> pv. <i>glycines</i> NCPPB 554	<i>Glycine max</i>	Sudan
[<i>X. campestris</i>] pv. <i>malloti</i> NCPPB 4347	<i>Mallotus japonicus</i>	Japan
<i>X. citri</i> subsp. <i>malvacearum</i>		
<i>X. citri</i> subsp. <i>malvacearum</i> ICMP 217 ^T	<i>Gossypium</i> sp.	USA
[<i>X. campestris</i>] pv. <i>thespesiae</i> LMG 9057	<i>Thespesia populnea</i>	India
[<i>X. campestris</i>] pv. <i>leeana</i> NCPPB 2229	<i>Leea edgeworthii</i>	India
<i>X. axonopodis</i> pv. <i>bauhiniae</i> NCPPB 1335	<i>Bauhinia racemosa</i>	India
<i>X. axonopodis</i> pv. <i>cajani</i> NCPPB 573	<i>Cajanus cajan</i>	India
<i>X. axonopodis</i> pv. <i>punicae</i> NCPPB 466	<i>Punica granatum</i>	India
[<i>X. campestris</i>] pv. <i>azadirachtae</i> NCPPB 2388	<i>Melia indica</i>	India
[<i>X. campestris</i>] pv. <i>bilvae</i> NCPPB 3213	<i>Aegle marmelos</i>	India
<i>X. axonopodis</i> pv. <i>melhusii</i> NCPPB 994	<i>Tectona grandis</i>	India
<i>X. axonopodis</i> pv. <i>malvacearum</i> NCPPB 633	<i>Gossypium</i> sp.	Sudan
<i>X. axonopodis</i> pv. <i>desmodiilaxiflori</i> NCPPB 3086	<i>Desmodium laxiflorum</i>	India
<i>X. axonopodis</i> pv. <i>vitians</i> NCPPB 976	<i>Lactuca</i> sp.	USA
<i>X. fuscans</i>		
<i>X. fuscans</i> subsp. <i>fuscans</i> NCPPB 381 ^T	<i>Phaseolus vulgaris</i>	Canada
<i>X. axonopodis</i> pv. <i>rhynchosiae</i> NCPPB 1827	<i>Rhynchosia memnonia</i>	Sudan
<i>X. axonopodis</i> pv. <i>sesbaniae</i> NCPPB 582	<i>Sesbania aegyptiaca</i>	India
<i>X. axonopodis</i> pv. <i>vignaeradiatae</i> NCPPB 2058	<i>Vigna radiata</i>	Sudan
<i>X. axonopodis</i> pv. <i>vignicola</i> NCPPB 1838	<i>Vigna unguiculata</i>	USA
<i>X. fuscans</i> subsp. <i>aurantifolii</i> NCPPB 3236 ^T	<i>Citrus</i> sp.	Argentina
[<i>X. campestris</i>] pv. <i>aracearum</i> NCPPB 2832	<i>Xanthosoma sagittifolium</i>	Guadeloupe

Table 1. cont.

Strain	Host	Country
[<i>X. campestris</i>] pv. <i>merremiae</i> NCPPB 3114	<i>Merremia gangetica</i>	India
[<i>X. campestris</i>] pv. <i>thirumalacharii</i> NCPPB 1452	<i>Triumfetta pilosa</i>	India
[<i>X. campestris</i>] pv. <i>trichodesmae</i> NCPPB 585	<i>Trichodesma zeylanicum</i>	India
<i>Xanthomonas</i> sp. NCPPB 3956	<i>Triticum aestivum</i>	Pakistan
<i>X. axonopodis</i>		
<i>X. axonopodis</i> pv. <i>axonopodis</i> NCPPB 457 ^T	<i>Axonopus scoparius</i>	Colombia
<i>X. axonopodis</i> pv. <i>vasculorum</i> NCPPB 796	<i>Saccharum officinarum</i>	Mauritius
<i>X. melonis</i>		
<i>X. melonis</i> NCPPB 3434 ^T	<i>Cucumis melo</i>	Brazil
[<i>X. campestris</i>] pv. <i>lantanae</i> NCPPB 1455	<i>Lantana camara</i> var. <i>aculeata</i>	India
<i>X. arboricola</i>		
<i>X. arboricola</i> pv. <i>juglandis</i> NCPPB 411 ^T	<i>Juglans regia</i>	New Zealand
<i>X. arboricola</i> pv. <i>celebensis</i> NCPPB 1832	<i>Musa</i> sp.	New Zealand
<i>X. arboricola</i> pv. <i>corylina</i> NCPPB 935	<i>Corylus maxima</i>	USA
<i>X. arboricola</i> pv. <i>populi</i> NCPPB 4342	<i>Populus</i> sp.	Netherlands
<i>X. arboricola</i> pv. <i>pruni</i> NCPPB 416	<i>Prunus salicina</i>	New Zealand
<i>X. arboricola</i> pv. <i>fragariae</i> LMG 19145	<i>Fragaria ananassa</i>	Italy
[<i>X. campestris</i>] pv. <i>arracaciae</i> NCPPB 2436	<i>Arracacia xanthorrhiza</i>	Brazil
<i>Xanthomonas</i> sp. NCPPB 3200	<i>Chrysanthemum morifolium</i>	Kenya
<i>Xanthomonas</i> sp. NCPPB 3218	<i>Syzygium aromaticum</i>	Indonesia
<i>Xanthomonas</i> sp. NCPPB 2856	<i>Chrysanthemum morifolium</i>	Netherlands
<i>Xanthomonas</i> sp. NCPPB 2864	<i>Chrysanthemum morifolium</i>	Netherlands
<i>Xanthomonas</i> sp. NCPPB 2865	<i>Chrysanthemum morifolium</i>	Netherlands
<i>Xanthomonas</i> sp. NCPPB 2866	<i>Chrysanthemum morifolium</i>	Netherlands
<i>Xanthomonas</i> sp. NCPPB 1826	<i>Prunus domestica</i>	UK
[<i>X. campestris</i>] pv. <i>guizotiae</i> NCPPB 1932	<i>Guizotia abyssinica</i>	Ethiopia
[<i>X. campestris</i>] pv. <i>zantedeschiae</i> NCPPB 2978	<i>Zantedeschia aethiopica</i>	South Africa
<i>X. oryzae</i>		
<i>X. oryzae</i> pv. <i>oryzae</i> NCPPB 3002 ^T	<i>Oryza sativa</i>	India
<i>X. oryzae</i> pv. <i>oryzicola</i> NCPPB 1585	<i>Oryza sativa</i>	Malaysia
[<i>X. campestris</i>] pv. <i>leersiae</i> NCPPB 4346	<i>Leersia hexandra</i>	China
<i>X. vasicola</i>		
<i>X. vasicola</i> pv. <i>holcicola</i> NCPPB 2417 ^T	<i>Sorghum vulgare</i>	New Zealand
[<i>X. campestris</i>] pv. <i>musacearum</i> NCPPB 2005	<i>Ensete ventricosum</i>	Ethiopia
[<i>X. campestris</i>] pv. <i>arecae</i> NCPPB 2649	<i>Areca catechu</i>	India
<i>Xanthomonas</i> sp. NCPPB 1394	<i>Tripsacum laxum</i>	Sri Lanka
<i>Xanthomonas</i> sp. NCPPB 1395	<i>Tripsacum laxum</i>	Sri Lanka
<i>Xanthomonas</i> sp. NCPPB 1396	<i>Tripsacum laxum</i>	Sri Lanka
<i>Xanthomonas</i> sp. NCPPB 902	<i>Tripsacum laxum</i>	Sri Lanka
<i>X. hortorum</i>		
<i>X. hortorum</i> pv. <i>hederae</i> NCPPB 939 ^T	<i>Hedera helix</i>	USA
<i>X. hortorum</i> pv. <i>pelargonii</i> NCPPB 2985	<i>Pelargonium peltatum</i>	New Zealand
<i>X. hortorum</i> pv. <i>taraxaci</i> NCPPB 940	<i>Taraxacum kok-saghyz</i>	USA
<i>X. hortorum</i> pv. <i>carotae</i> NCPPB 425	<i>Daucus carota</i>	Canada
[<i>X. campestris</i>] pv. <i>nigromaculans</i> NCPPB 1935	<i>Arctium lappa</i>	Japan
<i>Xanthomonas</i> sp. NCPPB 3959	<i>Persea americana</i>	New Zealand
<i>Xanthomonas</i> sp. NCPPB 2933	<i>Calendula officinalis</i>	UK
<i>X. gardneri</i> NCPPB 881 ^T	<i>Lycopersicon esculentum</i>	Yugoslavia
<i>X. cynarae</i> NCPPB 4356 ^T	<i>Cynara scolymus</i>	France
<i>X. populi</i> NCPPB 2959 ^T	<i>Aplanobacter populi</i>	France
<i>X. campestris</i>		
<i>X. campestris</i> pv. <i>campestris</i> NCPPB 528 ^T	<i>Brassica oleracea</i> var. <i>gemmifera</i>	UK
<i>X. campestris</i> pv. <i>aberrans</i> NCPPB 2986	<i>Brassica oleracea</i> var. <i>capitata</i>	Australia
<i>X. campestris</i> pv. <i>armoraciae</i> NCPPB 347	<i>Iberis</i> sp.	Tanzania
<i>X. campestris</i> pv. <i>barbareae</i> NCPPB 983	<i>Barbarea vulgaris</i>	USA
<i>X. campestris</i> pv. <i>incanae</i> NCPPB 937	<i>Matthiola</i> sp.	USA

Table 1. cont.

Strain	Host	Country
<i>X. campestris</i> pv. <i>plantaginis</i> NCPPB 1061	<i>Plantago lanceolata</i>	Ethiopia
<i>X. campestris</i> pv. <i>raphani</i> NCPPB 1946	<i>Raphanus sativus</i>	USA
[<i>X. campestris</i>] pv. <i>papavericola</i> NCPPB 2970	<i>Papaver rhoeas</i>	USA
<i>Xanthomonas</i> sp. NCPPB 2345	<i>Cheiranthus cheiri</i>	UK
<i>Xanthomonas</i> sp. NCPPB 2586	<i>Brassica juncea</i>	India
<i>Xanthomonas</i> sp. NCPPB 2918	<i>Crambe</i> sp.	Malawi
<i>Xanthomonas</i> sp. NCPPB 2922	<i>Matthiola incana</i>	Zimbabwe
<i>Xanthomonas</i> sp. NCPPB 3883	<i>Crambe abyssinica</i>	USA
<i>Xanthomonas</i> sp. NCPPB 3884	<i>Crambe abyssinica</i>	USA
<i>Xanthomonas</i> sp. NCPPB 4037	<i>Nemesia</i> sp.	UK
<i>Xanthomonas</i> sp. NCPPB 4231	<i>Beta vulgaris</i>	UK
<i>Xanthomonas</i> sp. NCPPB 4232	<i>Beta vulgaris</i>	UK
<i>X. translucens</i>		
<i>X. translucens</i> pv. <i>translucens</i> NCPPB 973 ^T	<i>Hordeum vulgare</i>	USA
<i>X. translucens</i> pv. <i>arrhenatheri</i> NCPPB 3229	<i>Arrhenatherum elatius</i>	Switzerland
<i>X. translucens</i> pv. <i>cerealis</i> NCPPB 1944	<i>Bromus inermis</i>	USA
<i>X. translucens</i> pv. <i>graminis</i> NCPPB 2700	<i>Dactylis glomerata</i>	Switzerland
<i>X. translucens</i> pv. <i>phlei</i> NCPPB 3231	<i>Phleum pratense</i>	Norway
<i>X. translucens</i> pv. <i>phleipratensis</i> NCPPB 1837	<i>Phleum pratense</i>	USA
<i>X. translucens</i> pv. <i>poae</i> NCPPB 3230	<i>Poa trivialis</i>	Switzerland
<i>X. translucens</i> pv. <i>secalis</i> NCPPB 2822	<i>Secale cereale</i>	Canada
<i>X. translucens</i> pv. <i>undulosa</i> NCPPB 2821	<i>Triticum durum</i>	Canada
[<i>X. campestris</i>] pv. <i>gummisudans</i> NCPPB 2182	<i>Gladiolus</i> sp.	Netherlands
<i>Xanthomonas</i> sp. NCPPB 3957	<i>Hordeum vulgare</i>	Zambia
<i>X. sacchari</i>		
<i>X. sacchari</i> LMG 471 ^T	<i>Saccharum officinarum</i>	Guadeloupe
[<i>X. campestris</i>] pv. <i>cannae</i> NCPPB 4345	<i>Canna</i> × <i>generalis</i>	India
<i>Xanthomonas</i> sp. NCPPB 1132	<i>Musa banksii</i> var. <i>samoensis</i>	Western Samoa
<i>Xanthomonas</i> sp. NCPPB 3174	<i>Setaria italica</i>	India
<i>Xanthomonas</i> sp. NCPPB 3570	<i>Xanthosoma sagittifolium</i>	Venezuela
<i>Xanthomonas</i> sp. NCPPB 888	<i>Saccharum officinarum</i>	South Africa
<i>Xanthomonas</i> sp. NCPPB 917	<i>Saccharum officinarum</i>	Trinidad
<i>X. cucurbitae</i>		
<i>X. cucurbitae</i> NCPPB 2597 ^T	<i>Cucurbita maxima</i>	New Zealand
<i>Xanthomonas</i> sp. NCPPB 1638	<i>Cucurbita pepo</i> var. <i>melopecto</i>	Uganda
<i>X. codiae</i>		
<i>X. codiae</i> NCPPB 4350 ^T	<i>Codiaeum variegatum</i>	USA
<i>Xanthomonas</i> sp. NCPPB 3581	<i>Strelitzia reginae</i>	USA
<i>Xanthomonas</i> sp. NCPPB 3582	<i>Strelitzia reginae</i>	USA
Slc 1		
[<i>X. campestris</i>] pv. <i>zinniae</i> NCPPB 2439	<i>Zinnia elegans</i>	Australia
[<i>X. campestris</i>] pv. <i>cannabis</i> NCPPB 2877	<i>Cannabis sativa</i>	Romania
<i>Xanthomonas</i> sp. NCPPB 1068	<i>Nicotiana tabacum</i>	Malawi
<i>Xanthomonas</i> sp. NCPPB 1325	<i>Helianthus annuus</i>	Uganda
Slc 2		
[<i>X. campestris</i>] pv. <i>esculenti</i> NCPPB 2190	<i>Hibiscus esculentus</i>	Brazil
[<i>X. campestris</i>] pv. <i>eucalyptii</i> NCPPB 2337	<i>Eucalyptus maculata</i> var. <i>citriodora</i>	Australia
[<i>X. campestris</i>] pv. <i>laureliae</i> NCPPB 1155	<i>Laurelia novaezelandiae</i>	New Zealand
<i>Xanthomonas</i> sp. NCPPB 1560	<i>Lobelia erinus</i>	UK
<i>Xanthomonas</i> sp. NCPPB 1599	<i>Lobelia</i> sp.	UK
Slc 3		
<i>Xanthomonas</i> sp. NCPPB 1067	<i>Raphanus sativus</i>	Vanuatu
Slc 4		
[<i>X. campestris</i>] pv. <i>parthenii</i> NCPPB 3888	<i>Parthenium hysterophorus</i>	India
[<i>X. campestris</i>] pv. <i>badrii</i> NCPPB 571	<i>Xanthium strumarium</i>	India

Table 1. cont.

Strain	Host	Country
Slc 5 <i>Xanthomonas</i> sp. NCPPB 2654	<i>Phaseolus vulgaris</i>	UK
Slc 6 [<i>X. campestris</i>] pv. <i>phormiicola</i> NCPPB 2983	<i>Phormium tenax</i>	Japan
Slc 7 <i>Xanthomonas</i> sp. NCPPB 1046	<i>Saccharum officinarum</i>	India
<i>Xanthomonas</i> sp. NCPPB 1117	<i>Saccharum officinarum</i>	India
<i>Xanthomonas</i> sp. NCPPB 901	<i>Gossypium</i> sp.	India
<i>Xanthomonas</i> sp. NCPPB 916	<i>Saccharum officinarum</i>	Trinidad
<i>Xanthomonas</i> sp. NCPPB 1131	<i>Musa paradisiaca</i>	American Samoa
<i>Xanthomonas</i> sp. NCPPB 2250	<i>Zingiber officinale</i>	Mauritius

Cannabis sativa. Two [*X. campestris*] pathovars of laurel and eucalyptus, together with an unidentified xanthomonad from lobelia leaf spots, constitute slc 2. An unidentified isolate from *Raphanus sativa* (Chinese radish) constitutes slc 3 and two [*X. campestris*] pathovars of *Xanthium strumarium* and *Parthenium hysterophorus* isolated from India represent slc 4. An unidentified bean pathogen and [*X. campestris*] pv. *phormiicola*, which infects *Phormium tenax*, represent slc 5 and 6, respectively. Six unidentified xanthomonads from sugar cane, banana, rice, ginger and cotton constitute slc 7.

DISCUSSION

The overall phylogenetic structure of the genus determined from the previous *gyrB* study using type strains, and which places mainly monocot pathogens as the most ancestral species, was preserved in this study. Xanthomonads with sufficiently distinct sequences to be identified as novel species-level clades were a small proportion of the strains sequenced, and comprised exclusively poorly characterized [*X. campestris*] pathovars or unidentified strains. The phylogeny appears monophyletic, and the constituent species have probably diversified from a single lineage. The correct assignment of well-characterized pathovars to clades containing their respective or related reference pathotype strains confirms the accuracy of both the existing classification and the *gyrB* phylogeny. The inclusion of *X. hortorum*, *X. cynarae* and *X. gardneri* within the same clade has been identified in the previous *gyrB* study (Parkinson *et al.*, 2007). The *X. hortorum* pv. *taraxi* sequence further extends the diversity of this clade and has resulted in the inclusion of *X. populi*, which was previously a sister species in the clade.

By including all the pathovar reference strains, the phylogeny has provided a comprehensive overview of xanthomonad relatedness, which has enabled the classification of hitherto-unidentified or poorly characterized pathogens. Many [*X. campestris*] pathovars were assigned to species related to *X. axonopodis*. This is consistent with the broad grouping of some of these pathovars (Vauterin

et al., 1990) that used earlier methods with less resolution than *gyrB* sequencing. Unidentified xanthomonads were assigned to a diverse range of species and novel species-level clades. Pathogenicity studies are needed to assess the disease significance of these hitherto-unidentified xanthomonads, especially for representatives of slc 7, which occur principally from important monocot crops.

The locus was sufficiently discriminating to identify intra-species sequence variation; considerable diversity was identified within *X. translucens*, *X. arboricola*, *X. hortorum* and *X. campestris*, and most constituent pathovars were differentiated. The phylogeny has established the lineage between *X. axonopodis* and recently described related taxa, i.e. *X. citri*, *X. fuscans* and the *X. euvesicatoria* species complex, which was identified in the previous *gyrB* study. All the *X. axonopodis* pathovars grouped in clades containing these species, reflecting the relatedness of the taxa. Additional structure within these major clades was identified, including a sister clade to the *X. euvesicatoria* species complex, which comprised six pathovars, and differentiated the bean pathogens *X. fuscans* and *X. axonopodis* pv. *phaseoli*. Within *X. citri*, the phylogeny discriminated two subclades containing the subspecies *X. citri* subsp. *citri* and *X. citri* subsp. *malvacearum* (Schaad *et al.*, 2006). The *X. citri* subsp. *citri* clade included pathovars from a wide host range, including five taxa known to infect members of the Vitaceae, as well as the commercially important pathogens [*X. campestris*] pv. *mangiferaeindicae* and *X. axonopodis* pv. *glycines*. The *X. citri* subsp. *malvacearum* clade also includes pathovars from diverse hosts, but with several plant families included in the *X. citri* subsp. *citri* clade, mainly from India. The *X. fuscans* clade comprises pathovars largely from hosts in the Fabaceae but including *X. fuscans* pv. *aurantifolii* from *Citrus aurantifolia*. The *X. axonopodis* clade comprises just two pathovars, both from the Poaceae, namely *X. axonopodis* pv. *axonopodis* and the pathotype strain of *X. axonopodis* pv. *vasculorum*. The *X. melonis* clade has two members, the type strain of *X. melonis* and the pathotype strain of [*X. campestris*] pv. *lantanae*, from unrelated hosts from Brazil and India, respectively. Hence, these two lineages have not been subjected to the intense pathovar diversification seen in related clades isolated from India.

Fig. 1. Comprehensive xanthomonad *gyrB* phylogeny. A conventional phylogeny is provided as Supplementary Fig. S1. Tree construction used the neighbour-joining method. Note several clades with minimum sequence diversity. Strains with sequences dissimilar to known type strains are highlighted as species-level clades in Fig. 2. Clade assignment of strains is provided in Table 1. T, Type strain of indicated taxon; X.sp., unidentified *Xanthomonas* sp. strains with the NCPPB strain number given. Other strains are indicated as 'X.species.pathovar'; poorly characterized pathovars referred to in the text as [*X. campestris*] are indicated by asterisks. Accession numbers of sequences obtained in this study are given in Supplementary Table S1.

The host ranges of several other species have also been extended by this study. The *X. arboricola* clade includes [*X. campestris*] pv. *arracaciae*, [*X. campestris*] pv. *guizotiae* and [*X. campestris*] pv. *zantedeschiae* (which has a monocot host), together with unclassified strains from chrys-

anthemum and clove. The *X. oryzae* clade includes [*X. campestris*] pv. *leersiae* from *Leersia*, a close relative of rice. The *Xanthomonas vasicola* clade includes [*X. campestris*] pv. *musacearum*, currently of great economic significance in East Africa, [*X. campestris*] pv. *arecae* from betel palm and

Fig. 2. *gyrB* phylogenetic identification of novel species-level clades (slc 1–7). The plant host is indicated for unidentified *Xanthomonas* sp. (*X. sp.*) isolates that have not been assigned to a pathovar. All strains are pathotype strains. Poorly characterized *X. campestris* pathovars are indicated by square brackets. The tree was constructed using the neighbour-joining method. Bootstrap values were from 200 samplings.

an unclassified pathogen from the forage grass *Tripsacum laxum*. Strains from sugar cane and maize from southern Africa, originally classified as [*X. campestris*] pv. *vasculorum* but not included in this study, also have similar *gyrB* sequences to other members of this clade (Aritua *et al.*, 2008). They also had fatty acid profiles very similar to that of *X. vasicola* pv. *holcicola* (Dookun *et al.*, 2000). These strains were included in the study of Vauterin *et al.* (1995) and were placed as *X. vasicola* pv. *vasculorum* but, since pathogenicity test results were not included, the name was not correctly proposed. The *X. hortorum* clade includes [*X.*

campestris] pv. *nigromaculans* and unclassified strains from *Persea americana* (avocado) and *Calendula officinalis*. The *X. campestris* clade includes [*X. campestris*] pv. *papavericola* as well as unclassified strains from *Nemesia* sp., *Beta vulgaris* and various cruciferous hosts. The *X. translucens* clade includes [*X. campestris*] pv. *gummisudans*, from *Gladiolus* sp., the first host outside the Poaceae. The *X. sacchari* clade includes [*X. campestris*] pv. *cannae* and unclassified strains from *Setaria italica* (Poaceae), *Musa banksii* in the Musaceae and *Xanthosoma sagittifolium* in the Araceae. The *Xanthomonas codiae* clade includes unclassified strains from *Strelitzia reginae* in the Strelitziaceae.

As well as identifying sequence diversity, this study has revealed previously unrecognized close relatedness between many pathovars. Minimal sequence diversity was found in a large number of xanthomonads which constitute the three clades that contained the *X. euvesicatoria* species complex, *X. citri* and *X. fuscans*. Though these three clades contain type strains that are crop pathogens of tomato, pepper, alfalfa, citrus and bean (Jones *et al.*, 2004; Schaad *et al.*, 2006), our phylogeny indicates that they share close relatedness to 86 other pathovars that infect diverse plant species, including many weed and other species that were isolated in agricultural regions of India. The *X. euvesicatoria* species complex alone comprises 49 pathovars, most of which have identical sequences. Lack of sequence variation between pathovars indicates that rapid diversification has occurred, before mutations in *gyrB* sequences have had time to accumulate. The close sequence similarity within these three clades indicates that a large proportion of pathovars isolated from Indian agricultural systems have diversified relatively recently from three principal founding ancestral lineages. Weed species can be traded widely but indirectly, as crop-seed contaminants. Whilst importation of infected weeds may have accounted for some introductions of these pathogens into the region, the very large number of pathovars from India strongly suggests that this region was the geographical centre for their diversification. Low-intensity agriculture is associated with high weed densities and farming practices that are likely to transfer pathogens between plants. These conditions, together with crop trading, may have contributed to rapid pathovar development and dissemination. Diversification of these lineages probably dates from the beginning of modern agriculture in the subcontinent. The historical origins and spread of citrus canker from Asia, caused by *X. citri*, has been described recently (Janse, 2006). A study from multiple loci from *X. euvesicatoria* strains from pepper has produced a divergence date range of between 28 and 1432 years ago, which corresponds to the time that these plants have been domesticated and cultivated (Wichmann *et al.*, 2005). Analysis of further loci may provide an estimate for the date at which the Indian xanthomonad species and lineages diverged.

Other examples of pathovar diversification in specific regional angiosperm floras are apparent which are similar to the regional centres found in endemic *Ralstonia*

solanacearum phylotypes (Fegan & Prior, 2005). Some, but not all, xanthomonads within *X. arboricola* comprise pathovars that infect plants that grow in European woodland, i.e. hazel, *Prunus*, walnut and strawberry. Pathovars of *X. campestris* may also have diversified in field systems, but from a different geographical centre other than India. *Brassica* species are common weeds of agricultural field systems, and many species are hosts of *X. campestris*. Extensive characterization of *X. campestris* isolates from naturalized *Brassica* species in coastal California revealed evidence of pathovar and race diversification in several related populations, which were generally found to be restricted to hosts within specific localized geographical sites (Ignatov *et al.*, 2007). The high frequency of xanthomonad isolation from these weeds and their distinctiveness from other local agricultural populations indicate that discrete xanthomonad populations are maintained in localized weed populations. In this current study, the inclusion of field poppy, plantain and chard pathogens within *X. campestris* is consistent with pathovar diversification among non-*Brassica* arable weeds. Those xanthomonads within the *X. vasicola* clade that have identical sequences were all isolated from monocotyledonous plants grown as plantation species (i.e. banana, sugar cane, Areca palm and Guatemala grass, *Tripsacum laxum*) and may have diversified into these hosts since farmers have been growing them together. Finally, *X. translucens*, a species largely restricted to hosts from the Poaceae and which was found to comprise pathovars that were a mixture of both earlier and more recently diverged origins, has probably diversified and been maintained in grasslands.

The identification of a large number of closely related pathovars that infect a diverse range of plant species has provided a new insight into the ability and potential of xanthomonad lineages to diversify and exploit new plant hosts. These pathovars will provide an important resource for future studies aimed at relating changes in xanthomonad genetic composition to host-range diversification. All classifications based on relatedness raise the question as to what cut-off should be applied to distinguish a species. The phylogeny has provided a means of quantifying and visualizing relatedness between xanthomonads that has clarified their classification. Whilst in this report we have concentrated on determining the overall phylogenetic structure of the genus to aid identification, our data clearly indicate the need for emendation of species descriptions. This issue can be addressed once further loci have been analysed to support the clade structure determined in this single-locus analysis. The simplicity of the protocols used for *gyrB* phylogenetic identification will facilitate the wider adoption of this identification scheme.

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