CHARACTERIZATION AND IDENTIFICATION OF BACTERIA ISOLATED FROM *LOLIUM PERENNE* AND *POA ANNUA*

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ABSTRACT

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Bacterial wilt on *Lolium perenne* and *Poa annua* caused by *Xanthomonas translucens* is a major concern on golf courses in the United States. Bacteria were isolated from both *L. perenne* and *P. annua* suffering bacterial wilt and were confirmed as the causal agents via Koch's postulates. Generation time of each bacterial isolate was measured at 25^0 C, 30^0 C and 35^0 C, respectively. Temperature influence on disease severity on both *L. perenne* and *P. annua* were evaluated at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively. Results revealed that the shortest generation time of each bacterial isolate was measured at 35^0 C, bacterial isolates were more pathogenic to the host from which they were isolated and varieties in virulence did exist in both *L. perenne* bacterial isolates and *P. annua* bacterial isolates. A positive correlation between temperature and disease severity was discovered on both *L. perenne* and *P. annua*. Results also revealed that *L. perenne* bacterial isolate, r3, is different from other bacterial isolates at many aspects. The 16S rDNA identification using a 1276-bp 16S rDNA sequence and the multilocus sequence analysis (MLSA) using a 1803-bp concatenated sequence of four housekeeping genes (*gyrB*, *fusA*, *gapA* and *lepA*) demonstrated that the *L. perenne* bacterial isolates, r0 and r5, were *X. translucens pv. graminis* and the *P. annua* bacterial isolates, p0, p6 and p8, were *X. translucens pv. poae* and suggested that the *L. perenne* bacterial isolate, r3, may be a new *Xanthomonas* species.

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CHAPTER ONE

XANTHOMONAS TRANSLUCENS AND BACTERIAL WILT OF TURFGRASS

INTRODUCTION

Bacteria belonging to genus *Xanthomonas* are also called xanthomonads. Xanthomonads are gram-negative, straight, mostly yellow-pigmented rods (1.2-3.0 μm long by 0.4-1.0 μm wide) with a polar flagellum [1] and are active between -4^0 C and 37^0 C. Xanthomonads divide by simple binary fission and form yellow, mucoid and convex colonies on nutrient agar (NA). The yellow color stems from xanthomonadin, a carotenoid-like pigment, which not only is unique to genus *Xanthomonas* [2] but also is a useful chemotaxonomic marker of genus *Xanthomonas* [3]. Xanthomonadin plays a role for protecting *Xanthomonas* spp. from photodynamic damage [4, 5, 6]. Most xanthomonads produce xanthan, an extracellular polysaccharide, which renders solution a high degree of viscosity and is resistant to high temperatures, high salt concentrations and acid pH [29]. Xanthan gum is produced industrially on a large scale as a stabilizing, emulsifying and gelling agent in numerous commercial products, particularly in the food industry [30]. Bacteria belonging to genus *Xanthomonas* are strictly aerobic chemoorganotrophs and most of the strains are phytopathogenic [1]. Representatives of phytopathogenic *Xanthomonas* spp. occur in many climate regions and especially subtropical and tropical areas all over the world [7, 29]. The pathogens cause a variety of disease including wilt, necrosis, gummosis and vascular or parenchymatous disease on leaves, fruits or stems on diverse monocotyledonous and dicotyledonous plant families [8]. According to the most thorough study in this domain [1], the host range of genus *Xanthomonas* includes at least 268 dicotyl and 124 monocotyl plant species [29]. Bacterial belonging to genus *Xanthomonas* infect many economically important crops [29].

Among the most devastating of them are those pathogens affecting primary food crops, such as *X. oryzae* on rice [9] and *X. campestris pv. manihotis* on cassava [10]. Other important pathogens include *X. axonopodis pv. phaseoli* causing bacterial blight of bean, *X. campestris pv. glycines* causing bacterial pustule of soybean, citri, responsible for citrus canker, *X. campestris pv. vesicatoria* causing bacterial spot of pepper and tomato, *X. campestris pv. campestris* causing black rot of crucifers and *X. translucens pv. translucens* causing leaf streak and black chaff of small grains [29]. *X. translucens pv. graminis* is the causal agent of bacterial wilt disease of creeping bentgrass (*Agrostis stolonifera*) and perennial ryegrass (*Lolium perenne*) [11, 12] while *X. translucens pv. poae* is causal agent of bacterial wilt of annual bluegrass (*Poa annua*) [13].

SYMPTOMS AND EPIDEMIOLOGY

Xanthomonads cause a variety of disease including wilt, necrosis, gummosis and vascular or parenchymatous disease on leaves, fruits or stems on diverse monocotyledonous and dicotyledonous plant families. The bacterial strains belonging to species *X. translucens* are the common causal agents of bacterial disease of small grains and grasses and cause a relatively narrow range of symptoms including leaf streak, black chaff, necrosis and wilt.

Leaf streak is the typical symptom of bacterial disease of wheat (*Triticum aestivum*) and triticale (× *Triticosecale*). It consists of elongated, watersoaked brown lesions several centimeters long which show drops of exudate and shiny, thin scales on the leaf [14]. Lesions are first distinct, but coalesce to cover larger solid areas on the leaf [14]. Different environmental conditions may induce symptoms of bacterial streak [14]. *X. translucens* exhibits ice nucleation activity [15] and an epiphytic population can increase frost damage to wheat plants grown in high-attitude areas [16]. However, it is evident that *X. translucens* grows best on culture medium at a high temperature (30^0 C) and does not need frost to cause infection [14].

X. translucens is one of the causal agent of bacterial wilt of grass. Symptoms of general wilt, etiolation, chlorosis, and decline are observed on individual plants in irregular areas of the grass stand. [17]. *X. translucens* are abundant in organic debris, in soil, on the surface of foliage and on the surface of root. They usually invade the grass via injuries or wounds on leaves or root [18, 19, 20, 21]. The wounds could be caused by organisms, such as nematodes and fungi, or could be caused by artificial activities, such as traffic, mowing, aerification and topdressing [18, 19, 20, 21]. They can also invade grass tissues through natural openings, such as stomata and hydathodes, under very wet condition. For example, the bacteria in guttation droplets could be absorbed into grass tissue through stomata and hydathodes as the leaves dry. Bacteria will colonize and plug the xylem vessels once they enter the grass, which will disrupt the flow of water to the foliage. On golf course, the splash of rain and irrigation water, the traffic, the mowing, the aerification and the topdressing activities are the major ways for the spreading of bacterial wilt. In growth chamber, the infected grasses will wilt first. Leaves may turn blue-gray, lime-green or yellows. Necrosis sometimes appears at the tips of leaf while chlorosis always develops the on leaves and coalesce to cover larger area of the leaf. The infected grass will collapse and die at last. In field, etiolation and decline are the typical symptoms that usually observed. Schmidt (1989) [22] found bacteria could be kept in dry grasses for one year at room temperature. Channon and Hissett (1984) [20] reported that infection could take place through roots growing in infested soil. In this case, debris of diseased plants in soil can act as an inoculum source for primary infection [18]. For secondary infection, the pathogen-carrying plants with or without symptoms can be a source of inoculum [18]. Schmidt (1989) [22] reported difference in resistance among cultivars of grass. Wang et al. [18] also reported there was difference in susceptibility among individual plants. Schmidt (1989) [22] clearly indicated that the main means of transmission of *X. campestris pv. graminis* on

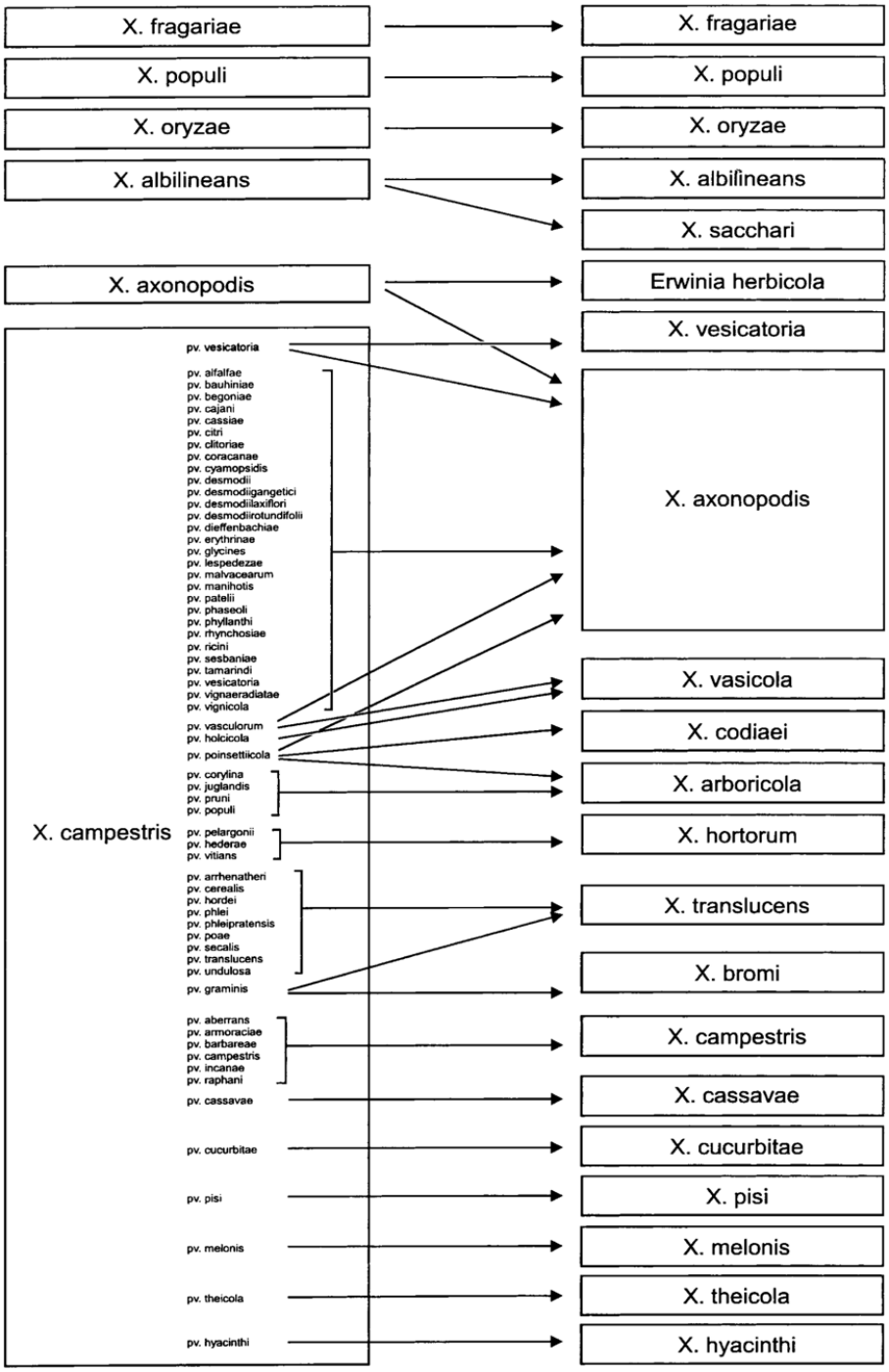
perennial ryegrass were mowing tools, young seedlings were particularly susceptible and very severe outbreak of bacterial wilt was related to extremely virulent bacterial strains.

XANTHOMONAS TAXONOMY

The most remarkable characteristics of genus *Xanthomonas* is the phytopathogenic diversity and the apparent host specificity of its members [26]. Originally, each variant showing a different host range or producing different disease symptoms was classified as a separate species [26]. It was denounced as the 'new host-new species' concept [7] which led to a complex genus containing more than 100 species [26]. In contrast to the phytopathogenic diversity of xanthomonads, the general phenotypic characteristics of xanthomonads are remarkably uniform [26]. Several comprehensive phenotypic studies have been performed in attempts to differentiate those phytopathogenic xanthomonads by means other than the host from which is isolated [28, 29, 31], but these studies only illustrated the phenotypic homogeneity of genus *Xanthomonas* [26]. This knowledge and the fact that insufficient information was available about the actual phytopathogenic specialization of the taxa, was the major motive for merging almost all *Xanthomonas* species into the single species *X. campestris* by Dye and Lelliott [31]. Later, Young *et al.* [32] proposed to reclassify the former *nomenspecies* (i.e., species only distinguished by their name) into pathovars, a pathovar is an infraspecific group which is defined only by the fact that it is, or is believed to be, characterized by a unique host range or disease [26]. Pathovar nomenclature was adopted as a provisional solution until a classification would be established based on more generally accepted principles. Thus, more than 140 pathovars have been defined within *X. campestris* [33, 34]. A series of studies on taxonomy of *Xanthomonas* have been undertaken to sort out the relationship between the many pathovars and species. Rather than extending classical phenotypic comparisons by testing individual biochemical and physiological features [35],

analytical fingerprinting techniques such as electrophoresis of whole protein [35] and gas-chromatographic analysis of cellular fatty acids [37] have been applied. Partial results based on protein electrophoresis applied on 307 *Xanthomonas* strains [38] have shown that the pathovars of *X. campestris* are much more heterogeneous than expected. Based upon cluster analysis of similarities between scanned and digitized protein patterns, 19 clusters could be delineated, of which *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*) was the most aberrant [26]. In some cases, pathovars from related hosts such as members of the plant families *Fabaceae*, *Poaceae*, and *Brassicaeae* seemed to be related to each other [39]. Similarly, quantitative comparison of cellular fatty acid content of more than 1000 strains in total demonstrated an unexpected high heterogeneity within *Xanthomonas*, especially among a number of pathovars of *X. campestris* [27,40]. The decisive information came from DNA homology measurements determined by DNA-DNA hybridization among 183 *Xanthomonas* strains, selected from both the protein and fatty acid groupings [11] and additional strains hybridized in other studies [41, 42]. Thus, the largest DNA homology matrix presently published allowed the distinction of 20 genomic groups [11]. Four groups contained respectively the existing species *X. albilineans*, *X. fragariae*, *X. populi*, and *X. oryzae*, whereas 16 DNA homology groups were new and not consistent with the existing pathovar classification [26]. The latter 16 genomic groups were consequently described as new species [11]. The complex rearrangements resulting from the DNA homology relationships within *Xanthomonas* are schematically represented in **Figure 1.1**.

Figure 1.1. Schematic representation of the rearrangements proposed within the genus *Xanthomonas*, resulting from a global taxonomic study of more than 1000 strains and DNA hybridization experiments between 183 selected strains [15] from Vauterin et al. (2000) [46] and supported by repetitive sequence-based polymerase chain reaction genomic fingerprinting [66, 67, 68, 69, 70].



Approved Lists of Bacterial Names [44] and International Code of Nomenclature [45] are two mandatory issues bacteriologists need to take into account with respect to propose nomenclatural changes of plant-pathogenic bacteria when a species is divided into two or more species. First, the specific epithet of the original species must be retained in one of the taxa into which the species is divided, and the specific epithet must be retained for the species which includes the type strain (rule 40a and 40b). Second, when two or more taxa of the same rank are united, then the name of the taxon under which they are united is chosen by the date of publication (rule 38)). Most of the reclassification by Vauterin et al. [11] were emendations from *X. campestris*, and the name *X. campestris* was reserved for the taxon that contains the type strain of that species [43]. In the cases of *cassavae*, *cucurbitae*, *pisi*, a part of *vesicatoria* (containing the pathovar reference strain), *hyacinthi*, *translucens*, *melonis*, and *theicola*, the species name was based upon the pathovar name, since they consist of only one homogeneous pathogenic group and the name is not misleading or confusing in terms of suggested host pathogenicity (*vesicatoria*, *translucens*) [43]. Identification and classification of *Xanthomonas translucens* variants (pathovars) pathogenic on members of the families *Gramineae* and *Poaceae* (cereals and grasses, respectively) is confusing [13]. The pathovar classification relied on the host range of the pathogen and is poorly defined due to variation in host-pathogen interactions [23]. Isolates with an unclear taxonomic position that display a distinct pathogenicity or obtained from novel hosts are often difficult to classify at subspecies level due to the limitations in examining a comprehensive host range [23]. Computer-assisted analysis of protein profiles has permitted the differentiation of pathovars *arrhenatheri*, *graminis*, *phlei*, *phleipratensis*, and *poae* [24], but not the other *X. translucens* pathovars [23]. In 1993, in serological tests using polyclonal and monoclonal antibodies, *X. translucens* pathovars *cerealis*, *hordei*, *secalis*, *translucens*, and *undulosa* form a homogeneous group [25]. In the 1995

revision of the genus *Xanthomonas* by Vauterin et al. [11], molecular analysis and other factors placed the pathovar of *Xanthomonas campestris* that infect grasses into the species *Xanthomonas translucens* [11].

INTRODUCTION OF PERENNIAL RYEGRASS AND ETIOLATION SYMPTOMS OF PERENNIAL RYEGRASS

Perennial ryegrass (*L. perenne*), also called English ryegrass, is a cool-season perennial bunchgrass native to Europe. It is widely distributed throughout the world, including in North and South America, Europe, New Zealand and Australia [46]. Perennial ryegrass is best adapted to cool, moist climates where winter kill is not a problem. It grows best on fertile, well-drained soils but has a wide range of soil adaptability and can tolerate both acidic and alkaline soils. Maximum growth of perennial ryegrass occurs between 68^0 F and 77^0 F (20 to 25^0 C), however, production of perennial ryegrass suffers when daytime temperatures exceed 87^0 F (31^0 C) and nighttime temperatures exceed 77^0 F (25^0 C), even with irrigation or abundant rainfall [46]. In United States, perennial ryegrass is used for forage predominately in the costal Northwest, irrigated intermountain valleys of the West, the Midwest and Northeast [46]. In United States, perennial ryegrass is also used for reducing soil erosion, for recycling nutrients from manure and as biosolids, wildlife feed and turf [46]. As great improvements in perennial ryegrass were made through breeding programs in late 1970s and 1980s, perennial ryegrass became the predominant fairway grass in the Mid-Atlantic region due to the improvements in shoot density, the ability to withstand the lower mowing heights demanded for fairways and the availability of endophyte-containing cultivars [47]. Cool-season turfgrasses always decline during the summer due to various factors including: heat stress, drought stress, soil temperature, other sources of natural physiological plant breakdown and biotic stress [48]. Gray leaf spot is a devastating disease which emerged in the 1990's on cool-season turfgrass [47]. Grey leaf spot is caused by the foliar fungal pathogen,

Pyricularia grisea [47]. It is devastating on ryegrass and can damage tall fescue [47]. Thus, this disease shifted golf courses towards using creeping bentgrass on fairways and other turf species in roughs [50]. The fungus, *P. grisea*, have been proposed to be associated with the perennial ryegrass summer decline complex as well [47].

The term etiolation is derived from the French etioler which means to grow pale and weak [49]. Etiolated tiller symptoms (ETS) was first coined by Jeff Gregos to describe the widespread epidemic of etiolated and damaged perennial ryegrass in fairways at Lebanon Country Club in North Cornwall, PA, during 2004 and 2005 [49]. Etiolation has been observed on annual bluegrass, creeping bentgrass and perennial ryegrass [50, 51]. Etiolation is the growth of shoots in the absence of light or in very low light which causes stems and leaves to become elongated and yellow due to the lack of chlorophyll [53]. However, low light conditions may not be the only reason for ETS, since symptoms have been observed in both shaded and full-sun areas on fairways, roughs, greens, green collars and approaches and tees [53]. It has been demonstrated that etiolation of annual bluegrass, creeping bentgrass and perennial ryegrass is caused by *X. translucens* spps. ETS usually appears during late spring to early summer and reappears in late summer to early fall [52]. The typical etiolated leaf blade of perennial ryegrass has an abnormal appearance of a yellow or light-green color accompanied by the abnormal growth of shoots and stems [52]. Frequently, only etiolated leaf blades are visible, particularly the youngest or newly emerged leaf blade, with no other turf damage visible [52]. An advanced stage resembles a “melting-out” turf appearance with a combination of etiolated leaf blades and collapse of surrounding tillers thinning out in patches or randomly, resulting in severe and necrotic turf damage [52]. ETS not only has been observed in United States but also has been observed around the world. [52].

HISTORY OF BACTERIAL WILT OF TURFGRASS IN THE UNITED STATES

Bacterial wilt, caused by *X. translucens pv. poae*, occurs primarily on golf course putting greens throughout the United States [54]. The disease most commonly causes pale, elongated growth referred to as etiolation and yellowing and progresses to cause foliar necrosis and plant death under favorable conditions [54].

Xanthomonas campestris pv. graminis was first described in 1981 in the lab of Dr. Joseph Vargas as the causal agent of bacterial wilt of Toronto C15 creeping bentgrass (*Agrostis stolonifera*). It was found that the bacterial wilt of Toronto C15 did not affect other cultivars of bentgrass nor annual bluegrass [55]. Bacterial wilt of annual bluegrass was originally identified in the United States in 1985, and the causal agent was considered a strain of *Xanthomonas campestris* [50]. The strain subsequently was named *X. campestris pv. poannua* [56, 57]. However, this name was not taxonomically recognized. In Japan, a pathogen identified as *X. campestris pv. poae* of annual bluegrass was found on putting greens of golf course [58, 59]. In 1995, a major revision of the genus *Xanthomonas* was published and reclassified those Xanthomonads that attack grasses as *Xanthomonas translucens*, a group of organisms that had been most previously revised under that name in the 1940s [60]. Mitkowski et al. [61] indicated the causal agent of bacterial wilt of annual bluegrass was *Xanthomonas translucens pv. poae*. Isolates infecting *A. stononifera* are known as *X. translucens pv. graminis*, and isolates infecting *P. annua* are *X. translucens pv. poae* based on the major revision of the genus *Xanthomonas* [11]. Bacterial wilt of perennial ryegrass (*L. perenne*) was first observed on a golf course fairway in Baltmore, MD, during hot (30^0 C to 33^0 C), humid weather in May 2013 [17]. The causal agent was successfully isolated and identified as *X. translucens pv. graminis* [17].

There are very few options available for controlling bacterial wilt of turfgrass. Although oxytetracycline was anecdotally shown to control bacterial wilt [50], this antibiotic is expensive, difficult to handle, phytotoxic and not labeled for use on turfgrass in the United States, therefore, cannot be used [51, 54, 62]. Copper can be used against bacterial wilt and it indeed reduces bacterial population on the surface of leaf [51]. However, copper is not effective against bacteria inside plant tissue, only the bacteria on the surfaces of leaf or in the soil will be affected [51]. So, copper is not widely used on golf course for controlling bacterial wilt. Cultural management methods are widely used for controling bacterial wilt. Mowing turf when leaves are dry, increasing mowing height, reducing mowing frequency, replacing grooved rollers with solid ones, disinfecting mowers, avoiding use of plant growth regulators, ceasing all grooming, aerification and topdressing practices when the disease is active, providing adequate ($0.125 lbs/1000 ft^2$) nitrogen fertility and frequent syringing and hand watering [51] are all management tactics aimed at reducing the disease or minimizing its spread.

Bacterial pathogens are rare and don't frequently cause prokaryotic disease on high value turfgrass. Thus, the focus on fungal pathogens in the turfgrass system may lead to misdiagnoses of maladies which are indeed caused by other biological organisms [60]. The newly identified bacterial wilt of perennial ryegrass was founded to be caused by *X. translucens pv. graminis*. The discovery, identification, characterization and progress of specific molecular diagnostics of the new bacterial turfgrass pathogen of perennial ryegrass are discussed herein.

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CHAPTER TWO

CHARACTERIZATION OF BACTERIA ISOLATED FROM *LOLIUM PERENNE* AND *POA ANNUA*

ABSTRACT

Bacterial wilt on *Lolium perenne* and *Poa annua* caused by *Xanthomonas translucens* is a major concern on golf courses in the United States. Bacteria were isolated from both *L. perenne* and *P. annua* suffering bacterial wilt and were confirmed as the causal agents via Koch's postulates. Generation time of each bacterial isolate was measured at 25^0 C, 30^0 C and 35^0 C, respectively. Temperature influence on disease severity on both *L. perenne* and *P. annua* were evaluated at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively. Results revealed that the shortest generation time of each bacterial isolate was measured at 35^0 C, bacterial isolates were more pathogenic to the host from which they were isolated and varieties in virulence did exist in both *L. perenne* bacterial isolates and *P. annua* bacterial isolates. A positive correlation between temperature and disease severity was discovered on both *L. perenne* and *P. annua*. Results also revealed that *L. perenne* bacterial isolate, r3, is different from other bacterial isolates.

INTRODUCTION

Bacterial wilt is a relatively new disease of turfgrass in North America [1]. In United States, bacterial wilt of creeping bentgrass (*Agrostis stolonifera cv. Toronto*) was originally identified in 1970s [2], bacterial wilt of annual bluegrass (*Poa annua*) was firstly reported in 1984 [1] and bacterial wilt of perennial ryegrass (*Lolium perenne*) was firstly reported in 2015 [3]. In the United States, the causal organisms of bacteria wilt of *A. stolonifera cv. Toronto*, *P. annua* and *L. perenne* were identified as *Xanthomonas translucens pv. graminis* [2], *Xanthomonas translucens pv. poae* [4] and *X. translucens pv. graminis* [3], respectively.

Bacterial wilt of turfgrass usually occurs from late spring through summer to early fall. Turfgrasses are predisposed to bacterial wilt by various stresses, such as intense mechanical activities (mowing and grooming, etc.), poor growing conditions (shade, poor air flow, poor water drainage and soil compaction, etc.) and environmental stresses (heat and drought, etc.). Bacterial wilt is favored by warm, rainy and overcast weather and is often triggered and intensified by heavy rains while is abated during dry periods but is flared up following rainfall. Under laboratory conditions, symptoms of bacterial wilt begin as lesions formed at the site of infection, typically the ends of the clipped leaves. Then, the tips of leaf will turn blue-green and begin to wilt. Then, chlorosis and necrosis begin to appear at the margin of leaves. Eventually, the grasses are dead. Under field conditions, general wilt, chlorosis and decline could be observed on individual plants in irregular area of the turf stand while etiolation is the most typical symptom.

Mitkowski et al. [4, 5] and Nishino et al. [6] demonstrated that bacterial wilt of *P. annua* could be easily induced by means of leaves clipping-inoculation. Leyns et al. [7] demonstrated that bacterial wilt could be easily induced by infecting *L. perenne* in leaves, roots and flowers and demonstrated that wilt symptoms were quickly induced when pathogen was inoculated near the leaf base. Wang et at [8] also demonstrated that bacterial wilt of *L. perenne* occurred mainly via wounds rather natural openings.

Egli et al. [9] in 1975 reported that the bacterial isolate of bacterial wilt from *Lolium multiflorum* was highly pathogenic to *L. multiflorum* and *L. perenne*. Egli and Schmidt in 1982 [10] reported that the *X. translucens pv. graminis* from *Lolium multiflorum* were highly pathogenic to *L. multiflorum* and *L. perenne* and mildly pathogenic to *Poa trivialis*. Egli and Schmidt [10] also reported that the *X. translucens pv. poae* from *Poa trivialis* were highly pathogenic to *P. trivialis* and intermediately pathogenic to *P. annua*. Egli and Schmidt [10] also reported a special case that

though only a few *L. multiflorum* were attacked, these attacks were particularly severe when *L. multiflorum* was infected with *X. translucens pv. poae* from *P. trivialis*. However, a few studies were conducted to study the host-parasite interaction between *L. perenne* and *P. annua*, respectively, and bacterial isolates of bacterial wilt from *L. perenne* and *P. annua*, respectively. The bacterial isolates used in the previous studies were restricted in Europe.

Imaizumi et al. [11] reported that the optimal growth temperature of liquid culture for *X. translucens pv. poae* was 30^0 C. Mitkowski et al. [4] also reported that the optimal growth temperature of liquid culture for *X. translucens pv. poae* was between 30^0 C and 35^0 C. Imaizumi et al. [11] reported that the generation time of *X. translucens pv. poae* in *P. annua* that was incubated at 25^0 C, 30^0 C and 35^0 C, respectively, was 8.25 h, 7.67 h and 7.88 h, respectively. However, Imaizumi et al. [11] didn't specify whether these generation times were significantly different from each other nor study the generation time of *X. translucens pv. poae* in liquid culture at 25^0 C, 30^0 C and 35^0 C, respectively. Few studies were conducted to study the optimal growth temperature for *X. translucens pv. graminis* in liquid culture and the generation times of *X. translucens pv. graminis* in liquid culture at different temperatures.

Imaizumi et al. [11] studied the effect of the environmental temperatures (including 25^0 C, 30^0 C and 35^0 C) on the development of the disease caused by *X. translucens pv. poae* on *P. annua* and demonstrated a positive correlation between temperature and fresh weight loss of host. Leyns et al. [12] studied the effect of the environmental temperatures (10^0 C, 18^0 C and 26^0 C) on the development of the disease caused by *X. translucens pv. graminis* on *L. perenne* and demonstrated an obviously positive correlation between temperature and disease severity. However, few studies were conducted to evaluate the effect of higher temperature on disease development on *L. perenne* infected with bacterial wilt.

In this study, bacteria were isolated from *L. perenne* (origin: US) infected with bacterial wilt and *P. annua* (origin: US) infected with bacterial wilt. Generation time of these bacterial isolates in liquid culture were measured at 25^0 C, 30^0 C and 35^0 C, respectively. Both *L. perenne* and *P. annua* were inoculated with *L. perenne* bacterial isolates and *P. annua* bacterial isolates, respectively, and incubated at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively. The objectives of this study were to 1) Elucidate the in-vitro generation times of *L. perenne* bacterial isolates and *P. annua* bacterial isolates at 25^0 C, 30^0 C and 35^0 C, respectively. 2) Elucidate the host range of *L. perenne* bacterial isolates and the host range of *P. annua* bacterial isolates. 3) Elucidate the optimal temperature for *L. perenne* bacterial isolates causing the most severe disease on *L. perenne* and *P. annua*, respectively, and the optimal temperature for *P. annua* bacterial isolates causing the most severe disease on *P. annua* and *L. perenne*, respectively. 4). Elucidate the pathogenicity of *L. perenne* bacterial isolates on *L. perenne* and *P. annua* and elucidate the pathogenicity of *P. annua* bacterial isolates on *P. annua* and *L. perenne*. 5). Elucidate the virulence varieties among *L. perenne* bacterial isolates and the virulence varieties among *P. annua* bacterial isolates.

MATERIALS AND METHODS

Bacterial isolation

Several samples of *L. perenne* infected with bacterial wilt were collected from different fairways of a golf course in Baltimore, MD and several samples of *P. annua* infected with bacterial wilt were collected from Hancock Turfgrass Research Center in East Lansing, MI. Bacterial streaming was present when cut ends of symptomatic leaves were observed with an Olympus BHB Phase Contrast Microscope at 100x (Olympus, Japan). These symptomatic leaves with bacterial streaming were used for bacterial isolation. For each sample, the causal agent was isolated by surface sterilizing of symptomatic leaves in 20% sodium hypochlorite solution for 3 mins followed

by a rinse in sterile phosphate buffer saline (PBS, pH=7.0) solution for 1 min. Then, the rinsed leaves were placed onto a 100 x 15 mm petri dish (VWR international, US) and were cut into 1-2 mm segments, and those leaf segments were soaked in 100 µL the sterile PBS for 5 mins. One hundred-fold serial dilutions up to 1 x 10^{-6} were performed in sterile PBS and 50 µl of each diluted suspension was plated on nutrient agar (NA, The NA was made from 7 g Nutrient Broth (Difco and BD, France), 23 g Bacto^{TM} Agar (BD, Sparks, MD, US) and 1 L distilled water) and incubated at 23 ^{0}C for 5 days. The most frequently observed colonies were yellow pigmented, mucoid and convex. Pure culture of causal agent was obtained by selecting a single-bacterial colony and growing it in *Xanthomonas* broth medium (XBM) (0.5 g K_2HPO_4, 0.5 g $NH_4H_2PO_4$, 0.2 g $MgSO_4 \bullet 7H_2O$, 5 g NaCl, 10g yeast extract (BD, Sparks, MD, US), 10 g sucrose 10 g, 1 L distilled water 1 L, final pH=7.0) for 5 days. For each *L. perenne* bacterial isolate, the suspension of the bacterial isolate was used to inoculate *L. perenne* grown in loam in a 296 ml styrofoam cup. For each *P. annua* bacterial isolate, the suspension of the bacterial isolate was used to inoculate *P. annua* grown in loam in a 296 ml styrofoam cup. The blades of sterile scissors were soaked in bacterial suspension and were used to trim the leaves of health plants. The inoculated plants were incubated at 23^{0} C. Inoculation with predominant *L. perenne* bacterial isolates (r0, r3 and r5) obtained from original *L. perenne* samples resulted in symptoms of wilt, necrosis and chlorosis on newly inoculated *L. perenne* and heavy bacterial streaming could be observed microscopically when symptomatic leaves of newly inoculated *L. perenne* were cut. Inoculation with predominant *P. annua* bacterial isolates (p0, p6 and p8) obtained from original *P. annua* samples resulted in symptoms of wilt, necrosis and chlorosis on newly inoculated *P. annua* and heavy bacterial streaming could be observed microscopically when symptomatic leaves of newly inoculated *P. annua* were cut. Isolation of r0, r3, r5, p0, p6 and p8 from their own newly inoculated hosts were

performed to fulfill the Koch's postulates. These six bacterial isolates were the pathogen used for the following study.

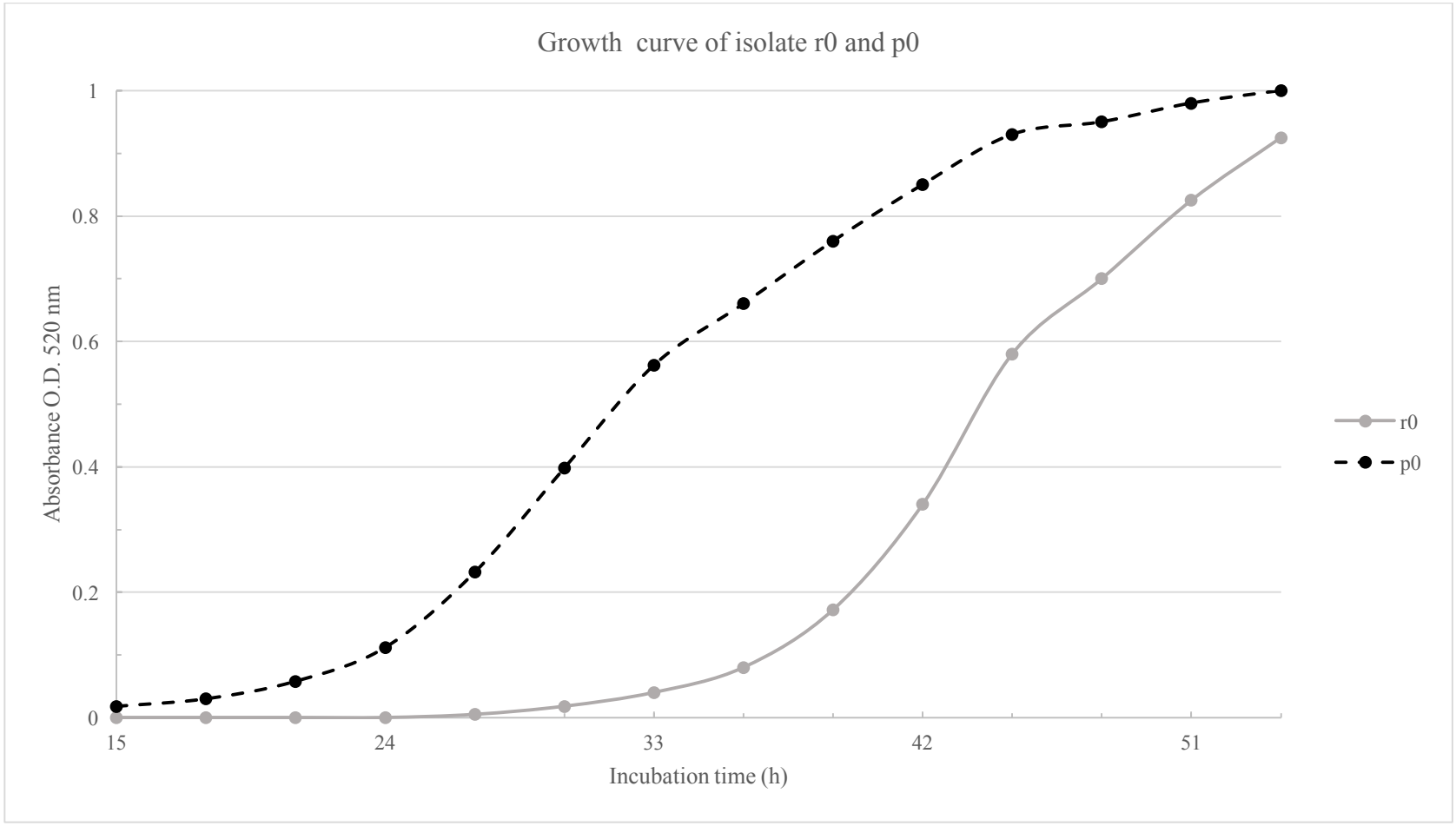
Hosts preparation

L. perenne and *P. annua* (*Poa annua cv. reptans*) were used in this study. Seeds were sown in 296 ml styrofoam cups filled with loam and incubated under standard greenhouse conditions (20^0 C to 25^0 C). Plants were daily watered with 24 mL distilled water and fertilized with 12 mL Peter's professional fertilizer (20-20-20) (ICL Specialty Fertilizers, Dublin, OH, US) at rate of 0.019 g nitrogen ml^{-1} every two weeks. Plants were grown for 4 weeks until pots were filled with complete plants coverage.

Bacterial growth curve

Two bacterial isolates (r0 and p0) were used for construction of bacterial growth curve. Single colony was transferred to 50 mL XBM and incubated at 23^0C. Three milliliter of the culture were collected every 3 h and centrifuged for 3 min in a Micro Centrifuge model 235C (Fisher Scientific, Hampton, NH, US) setting at 12400 rpm. Supernatant was discarded and pellet was suspended in 3 ml sterile PBS. Absorbance was measured using a SPECTRONIC 20 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, US) setting at 520 nm. Serial dilutions (final concentration: 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}) were performed, 50 µL of each dilution were plated on NA and incubated at 23^0C for 5 days. Colonies numbering between 30-200 were counted and recorded. The bacterial growth curve was listed in **Figure 2.1**.

Figure 2.1. Growth curve of *Lolium perenne* bacterial isolate, r0, and *Poa annua* bacterial isolate, p0*,* in *Xanthomonas* broth media at 23^0 C.



Bacterial generation time

The six bacterial isolates (r0, r3, r5, p0, p6 and p8) were used in this study. Test tubes containing 3 ml of sterile XBM were inoculated with pure colonies of bacteria from NA and were incubated on a G24 ENVIRONMENTAL INCUBATOR SHAKER (New Brunswick Scientific Co Inc, Edison, N. J., US) (100 rpm) at 25^0 C, 30^0 C and 35^0 C, respectively for 12 h. Then, the inoculated XBM was collected every 4h (240 min) and collected twice in total. Then the collected XBM was diluted 10^6 times and 20 μL of the diluted XBM was plated on NA and incubated at 23^0 C for 5 days. Numbers of bacterial colonies were counted and recorded and generation time of bacterial isolate was estimated by using the following equation: $G=t/(3.3\log_{10}(b/B)$ (G: generation time of bacterial isolate, t: time interval, 4 h (240 min), b: number of bacterial colonies at the end of the time interval, B: number of the bacterial colonies at the beginning of the time interval). The experiment was repeated 3 times.

Temperature tests

Bacteria were streaked on NA and incubated at 23^0 C for 5 days. Single colony was transferred to 50 mL XBM in a 150-mL flask and incubated at 23^0 C on a SK-71 shaker (Jeio tech inc, Billerica, MA, US) setting at 100 rpm for 48 h. Bacterial culture was centrifuged for 6 min in a Sorvall Legen RT Centrifuge (Kendro Laboratory Products Inc, Asheville, NC, US) setting at 3760 X g. Supernatant was discarded and pellet was suspended in 50 mL sterile PBS. After vortexing, absorbance was measured via a SPECTRONIC 20 spectrophotometer setting at 520 nm and bacterial concentration was adjusted to 10^9 cells mL^{-1} ($Log_{10}CFU=9$). Hosts were placed in a growth chamber and incubated for 24 h before inoculation. One milliliter of bacterial suspension was transferred to the blades of sterile scissors which were used to trim the plants. A total of 5 mL of bacterial suspension were used for each cup of plants and plants were trimmed to a final height

of 3 cm. Control plants were treated similarly but trimming with sterile scissors soaked with the sterile PBS. Each treatment has four replicates and experiment was repeated twice. After inoculation, plants were transferred to a Conviron HL-E8VH growth chamber. Plants were watered daily with 24 mL distilled water.

Disease severity was measured every three days. The severity of wilt symptom was evaluated via 0-0.5 visual scale. The 0 represents no area on the individual plant showing wilt symptom. The 0.25 represents half of the area on the individual plant showing wilt symptom. The 0.5 represents all the area on the individual plant showing wilt symptom. The severity of chlorosis symptom was also evaluated via 0-0.5 visual scale. The 0 represents no area on the individual plant showing chlorosis symptom. The 0.25 represents half of the area on the individual plant showing chlorosis symptom. The 0.5 represents all the area on the individual plant showing chlorosis symptom. The severity value of wilt symptom and the severity value of chlorosis were summed up to a 0-1 scale. The 0-1 scale was used to describe the disease severity. Data analysis was performed using ANOVA and given a significant ANOVA p-value ($P<0.01$) in SAS university edition.

RESULTS

Bacterial host range

Bacterial streaming oozing from the leaves cut of both inoculated *L. perenne* and inoculated *P. annua*, which confirmed successful infection, was observed under 100X power Olympus BHB Phase Contrast Microscope (Olympus, Japan) one day post inoculation. The results revealed that both the three *L. perenne* bacterial isolates (r0, r3 and r5) and the three *P. annua* bacterial isolates (p0, p6 and p8) could infected both *L. perenne* and *P. annua*.

Bacterial generation time

For each of the six bacterial isolates (r0, r3, r5, p0, p6 and p8), the shortest generation time was

measured at 35^0 C **Table 2.1 (a)**. However, for each of the six bacterial isolates, the generation time at 25^0 C was not significantly different from the generation time at 30^0 C (P>0.01) **Table 2.1 (a)**.

There was no significant difference among the generation times of the six bacterial isolates when they were incubated at 25^0 C, 30^0 C and 35^0 C, respectively (P>0.01) **Table 2.1 (b)**.

Table 2.1 (a, b). **(a)**. The generation time of each of the six bacterial isolates (r0, r3, r5, p0, p6 and p8) at each of three incubation temperatures (25^0 C, 30^0 C and 35^0 C). **(b)**. Pairwise comparison of generation times of the six bacterial isolates (r0, r3, r5, p0, p6 and p8) at each of the three incubation temperatures (25^0 C, 30^0 C and 35^0 C).

(a).

Isolates	Temperatures	Generation Times (Minutes)
r0	25℃	67.6
r0	30℃	64.6
r0	35℃	53.9*
r3	25℃	72.4
r3	30℃	65.5
r3	35℃	57.1*
r5	25℃	72.6
r5	30℃	66.9
r5	35℃	58.0*
p0	25℃	70.3
p0	30℃	67.7
p0	35℃	55.7*
p6	25℃	68.4
p6	30℃	65.2
p6	35℃	55.7*
p8	25℃	71.0
p8	30℃	70.8
p8	35℃	58.5*

For each of the six bacterial isolates, the generation time with "*" represents that it is significantly different from other generation times. Significant difference was determined according to Least Significant Difference multiple comparison test ($P<0.01$).

(b).

Temperatures	Generation Times (Minutes) of Isolates
25℃	Gr0(a), Gr3(a), Gr5(a), Gp0(a), Gp6(a), Gp8(a)
30℃	Gr0(a), Gr3(a), Gr5(a), Gp0(a), Gp6(a), Gp8(a)
35℃	Gr0(a), Gr3(a), Gr5(a), Gp0(a), Gp6(a), Gp8(a)

Gr0, Gr3, Gr5, Gp0, Gp6 and Gp8 represent the generation time of r0, r3, r5, p0, p6 and p8, respectively. Generation times with same little letter in bracket are not significantly different from each other. Significant difference was determined according to Least Significant Difference multiple comparison test ($P<0.01$).

Temperature tests

Disease development and disease severity: *Lolium perenne* infected with *Lolium perenne* bacterial isolates (r0, r3 and r5) and *Poa annua* bacterial isolates (p0, p6 and p8)

At 25/18^0 C, for *L. perenne* inoculated with *L. perenne* bacterial isolates, r0 and r5 respectively, significant disease was developed on *L. perenne* 6, 9, 12 and 15 dpi (days post inoculation) when compared to PBS control (P<0.01, <0.01, <0.01 and <0.01, respectively) **Table 2.2 (a)**, **Figure 2.2 (a)**. At 25/18^0 C, for *L. perenne* inoculated with *L. perenne* bacterial isolate, r3, significant disease was developed on *L. perenne* 9, 12 and 15 dpi when compared to PBS control (P<0.01, <0.01 and <0.01, respectively) **Table 2.2 (a), Figure 2.2 (a)**.

At 25/18^0 C, for *L. perenne* inoculated with *P. annua* bacterial isolates, p0, p6 and p8, respectively, significant disease was developed on *L. perenne* 9, 12 and 15 dpi when compared to PBS control (P<0.01, 0.01 and 0.01, respectively) **Table 2.2 (a), Figure 2.2 (a)**.

At 30/23^0 C, for *L. perenne* inoculated with *L. perenne* bacterial isolates, r0 and r5, and *P. annua* bacterial isolates, p0, p6 and p8, respectively, significant disease was developed on *L. perenne* 6, 9, 12 and 15 dpi when compared to PBS control (P<0.01, <0.01, <0.01 and <0.01, respectively) **Table 2.2 (a), Figure 2.2 (b)**. At 30/23^0 C, for the *L. perenne* inoculated with *L. per*enne bacterial isolate, r3, significant disease was developed on *L. perenne* 6, 9 and 12 dpi when compared to PBS control (P<0.01, <0.01 and <0.01, respectively) **Table 2.2 (a), Figure 2.2 (b)**. However, *L. perenne* bacterial isolate, r3, failed to cause significant disease on *L. perenne* 15 dpi when compared to PBS control (P=0.0139) **Table 2.2 (a), Figure 2.2 (b)**.

At 35/28^0 C, for *L. perenne* inoculated with *L. perenne* bacterial isolates, r0 and r5, and *P. annua* bacterial isolates, p0 and p8, respectively, significant disease was developed on *L. perenne* 6, 9, 12 and 15 dpi when compared to PBS control (P<0.01, <0.01, <0.01 and <0.01, respectively) **Table 2.2 (a), Figure 2.2 (c)**. At 35/28^0 C, for *L. perenne* inoculated with *L.*

perenne bacterial isolate, r3, and *P. annua* bacterial isolate, p6, respectively, significant disease was developed on *L. perenne* 12 and 15 dpi when compared to PBS control (P<0.01 and <0.01, respectively) **Table 2.2 (a), Figure 2.2 (c)**.

At $25/18^0$ C, comparing to *P. annua* bacterial isolates, the *L. perenne* bacterial isolates, r0 and r5, caused significantly more severe disease on *L. perenne* (P<0.01) **Table 2.2 (b)** while *L. perenne* bacterial isolates, r3, failed to cause significantly more severe disease on *L. perenne* (P> 0.01) **Table 2.2 (b)**.

At $30/23^0$ C, comparing to *P. annua* bacterial isolates, *L. perenne* bacterial isolates, r0 and r5, caused significantly more severe disease on *L. perenne* (P<0.01) **Table 2.2 (b)** while *L. perenne* bacterial isolates, r3, failed to cause significantly more severe disease on *L. perenne* (P>0.01) **Table 2.2 (b)**.

At $35/28^0$ C, comparing to *P. annua* bacterial isolates, p6 and p8, *L. perenne* bacterial isolates, r0 and r5, caused significantly more sever disease on *L. perenne* (P<0.01) **Table 2.2 (b)**. However, comparing to *P. annua* bacterial isolate, p0, *L. perenne* bacterial isolates, r0 and r5, failed to cause significantly more severe disease on *L. perenne* (P>0.01) **Table 2.2 (b)**. At $35/28^0$ C, comparing to *P. annua* bacterial isolates, *L. perenne* bacterial isolate, r3, failed to cause significantly more severe disease on *L. perenne* (P>0.01) **Table 2.2 (b)**.

These results indicate that, comparing to *L. perenne* bacterial isolates, r0 and r5, a delay in disease development was discovered on *L. perenne* when *L. perenne* was infected with *P. annua* bacterial isolates. These results indicate that, comparing to *L. perenne* bacterial isolates, r0 and r5, a delay in disease development was discovered on *L. perenne* when *L. perenne* was infected with *L. perenne* bacterial isolate, r3. These results indicate that *L. perenne* is more susceptible to *L. perenne* bacterial isolates, r0 and r5, than all three *P. annua* bacterial isolates at $25/18^0$ C and

30/23^0 C and *L. perenne* is more susceptible to *L. perenne* bacterial isolates, r0 and r5, than *P. annua* bacterial isolates, p6 and p8, at 35/28^0 C. These results indicate that *L. perenne* is as susceptible to *P. annua* bacterial isolate, p0, as to *L. perenne* bacterial isolates, r0 and r5, at 35/28^0 C. These results also indicate that comparing to *P. annua* bacterial isolates, the *L. perenne* bacterial isolate, r3, was not more pathogenic to *L. perenne*.

Disease development and disease severity: *Poa annua* infected with *Poa annua* bacterial isolates (p0, p6 and p8) and *Lolium perenne* bacterial isolates (r0, r3 and r5)

At 25/18^0 C, for *P. annua* inoculated with *P. annua* bacterial isolates, p0, p6 and p8, respectively, significant disease was developed on *P. annua* 6, 9, 12 and 15 dpi when compared to PBS control (P<0.01, <0.01, <0.01 and <0.01, respectively) **Table 2.2 (a), Figure 2.2 (d)**.

At 25/18^0 C, for *P. annua* inoculated with *L. perenne* bacterial isolates, r0 and r3, respectively, significant disease was developed on *P. annua* 12 and 15 dpi when compared to PBS control (P<0.01 and <0.01, respectively) **Table 2.2 (a), Figure 2.2 (d)**. At 25/18^0 C, for *P. annua* inoculated with *L. perenne* bacterial isolates, r5, significant disease was developed on *P. annua* 9, 12 and 15 dpi when compared to PBS control (P<0.01, <0.01 and <0.01, respectively) **Table 2.2 (a), Figure 2.2 (d)**.

At 30/23^0 C, for *P. annua* inoculated with *P. annua* bacterial isolates, p0, p6 and p8, respectively, significant disease was developed on *P. annua* 6, 9, 12 and 15 dpi when compared to PBS control (P<0.01, <0.01, <0.01 and <0.01, respectively) **Table 2.2 (a), Figure 2.2 (e)**.

At 30/23^0 C, for *P. annua* inoculated with *L. perenne* bacterial isolates, r0, r3 and r5, respectively, significant disease was developed on *P. annua* 9, 12 and 15 dpi when compared to PBS control (P<0.01, 0.01 and <0.01, respectively) **Table 2.2 (a), Figure 2.2 (e)**.

At 35/28^0 C, for *P. annua* inoculated with *P. annua* bacterial isolates, p0, p6 and p8, respectively, significant disease was developed on *P. annua* 6, 9, 12 and 15 dpi when compared

to PBS control ($P<0.01$, <0.01, <0.01 and <0.01, respectively) **Table 2.2 (a), Figure 2.2 (f)**.

At $35/28^0$ C, for *P. annua* inoculated with *L. perenne* bacterial isolates, r0, r3 and r5, respectively, significant disease was developed on *P. annua* 9, 12 and 15 dpi when compared to PBS control ($P<0.01$, <0.01 and <0.01, respectively) **Table 2.2 (a)**, **Figure 2.2 (f)**.

Comparing to *L. perenne* bacterial isolates, *P. annua* bacterial isolates caused significantly more severe disease on *P. annua* at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively **Table 2.2 (b)**.

These results indicate that, comparing to *P. annua* bacterial isolates, a delay in disease development was discovered on *P. annua* when *P. annua* was infected with *L. perenne* bacterial isolates. These results also indicate that *P. annua* is more susceptible to the three *P. annua* bacterial isolates (p0, p6 and p8) than the three *L. perenne* bacterial isolates (r0, r3 and r5) at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively.

Table 2.2 (a, b). **(a)**. Disease development on *Lolium perenne* and *Poa annua* after inoculating with *Lolium perenne* bacterial isolates r0, r3 and r5, and *Poa annua* bacterial isolates, p0, p6 and p8, respectively, as influenced by temperatures. **(b)**. Difference between disease severity on hosts inoculated with *Lolium bacterial* isolates (r0, r3, r5) and disease severity on hosts inoculated with *Poa annua* bacterial isolates (p0, p6 p8) at same temperature.

(a).

Hosts	Isolates	25/18 Degree of Celsius					30/25 Degree of Celsius					35/28 Degree of Celsius				
		3 dpi	6 dpi	9 dpi	12 dpi	15 dpi	3 dpi	6 dpi	9 dpi	12 dpi	15 dpi	3 dpi	6 dpi	9 dpi	12 dpi	15 dpi
***Lolium perenne* (R)**	r0		*	*	*	*		*	*	*	*		*	*	*	*
	r3			*	*	*		*	*	*					*	*
	r5		*	*	*	*		*	*	*	*		*	*	*	*
***Lolium perenne* (R)**	p0			*	*	*		*	*	*	*		*	*	*	*
	p6			*	*	*		*	*	*	*				*	*
	p8			*	*	*		*	*	*	*		*	*	*	*
***Poa annua* (P)**	p0		*	*	*	*		*	*	*	*		*	*	*	*
	p6		*	*	*	*		*	*	*	*		*	*	*	*
	p8		*	*	*	*		*	*	*	*		*	*	*	*
***Poa annua* (P)**	r0				*	*			*	*	*			*	*	*
	r3				*	*			*	*	*			*	*	*
	r5			*	*	*			*	*	*			*	*	*

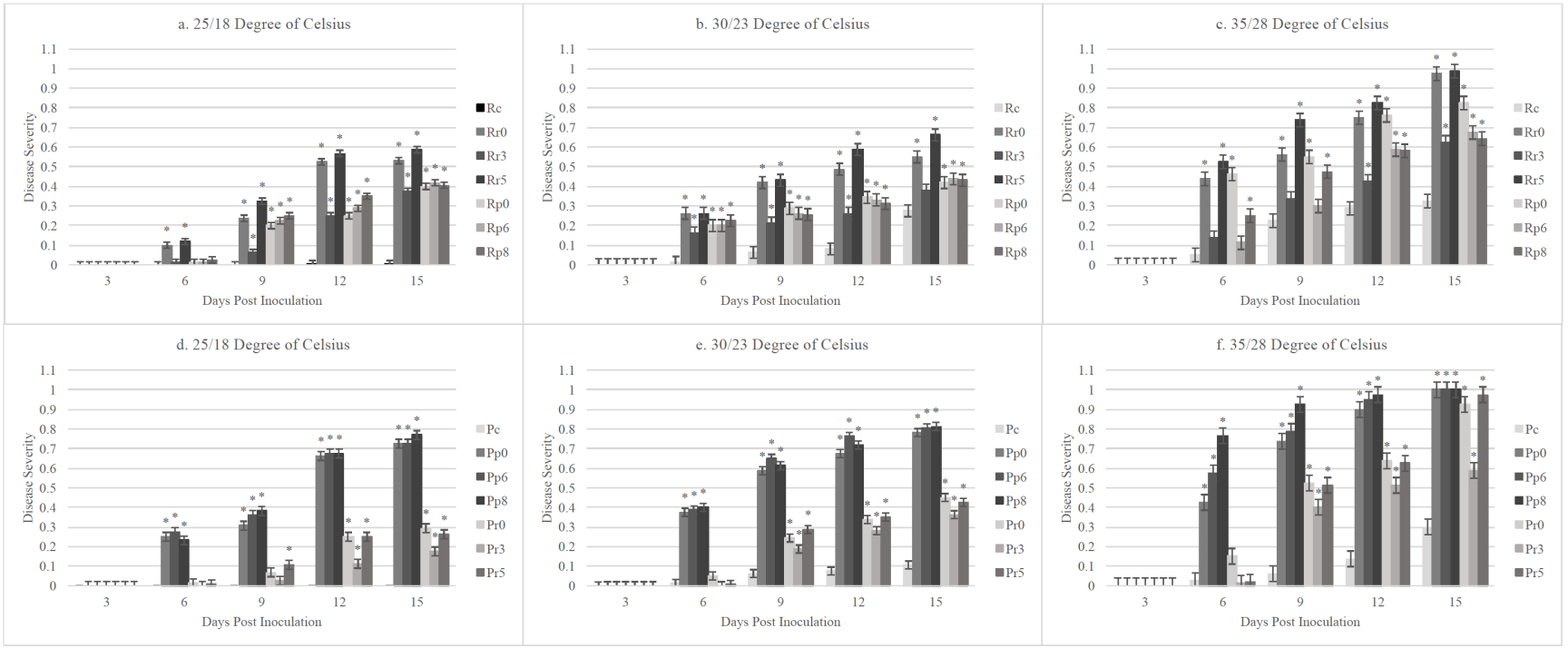
"dpi" represents "days post inoculation".
Within rating day and assessment, "*" represents a significant difference between treatments and control according to Least Significant Difference multiple comparison test (P<0.01).

(b).

Host	25/18 Degree of Celsius									30/23 Degree of Celsius									35/28 Degree of Celsius								
	r0-p0	r0-p6	r0-p8	r3-p0	r3-p6	r3-p8	r5-p0	r5-p6	r5-p8	r0-p0	r0-p6	r0-p8	r3-p0	r3-p6	r3-p8	r5-p0	r5-p6	r5-p8	r0-p0	r0-p6	r0-p8	r3-p0	r3-p6	r3-p8	r5-p0	r5-p6	r5-p8
***Lolium perenne* (R)**	+	+	+	-	-	-	+	+	+	+	+	+	=	=	=	+	+	+	=	+	+	-	=	-	=	+	+
	p0-r0	p0-r3	p0-r5	p6-r0	p6-r3	p6-r5	p8-r0	p8-r3	p8-r5	p0-r0	p0-r3	p0-r5	p6-r0	p6-r3	p6-r5	p8-r0	p8-r3	p8-r5	p0-r0	p0-r3	p0-r5	p6-r0	p6-r3	p6-r5	p8-r0	p8-r3	p8-r5
***Poa annua* (P)**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

"+" represents significantly positive, "-" represents significantly negative and "=" represents no significant difference.
For example, "r0-p0" represents the difference between disease severity on host infected with r0 and disease severity on host infected with p0. Significant difference was determined according to Least Significant Difference multiple comparison test (P<0.01).

Figure 2.2 (a-f). Disease development and severity of *Lolium perenne* and *Poa annua* after inoculating with *Lolium perenne* bacterial isolates r0, r3 and r5, respectively, and *Poa annua* bacterial isolates, p0, p6 and p8, respectively, as influenced by temperatures.



Error bars represent standard error of mean (n=4).

Rc and Pc represent *Lolium perenne* inoculated with sterile PBS (blank control) and *Poa annua* inoculated with sterile PBS (blank control), respectively. Rr0, Rr3, Rr5, Rp0, Rp6 and Rp8 represent *Lolium perenne* inoculated with bacterial isolates r0, r3, r5, p0, p6 and p8, respectively. Pr0, Pr3, Pr5, Pp0, Pp6 and Pp8 represent *Poa annua* inoculated with bacterial isolates r0, r3, r5, p0, p6 and p8, respectively.

Within rating day and assessment, "*" represents a significant difference between treatments and control according to Least Significant Difference multiple comparison test ($P<0.01$).

Temperature influence on *Lolium perenne* inoculated with sterile PBS and *Poa annua* inoculated with sterile PBS.

Both *L. perenne* and *P. annua* showed few wilt symptom at $30/23^0$ C and $35/28^0$ C, respectively, while were looked healthily at 25^0 C. The wilt symptom was significantly more obvious on both *L. perenne* and *P. annua*, respectively, at $35/28^0$ C than $30/23^0$ C and the wilt symptom was significantly more obvious on both *L. perenne* and *P. annua*, respectively, at $30/23^0$ C than $25/18^0$ C.

The wilt symptom was significantly more obvious on *L. perenne* than *P. annua* at $35/28^0$ C while the wilt symptom on *L. perenne* was not significantly different from the wilt symptom on *P. annua* at $25/18^0$ C and $30/23^0$ C, respectively.

Temperature influence on disease severity: *Lolium perenne* and *Poa annua* infected with *Lolium perenne* bacterial isolates (r0, r3 and r5)

For *L. perenne* that inoculated with *L. perenne* bacterial isolates, r0, r3 and r5, respectively, the significantly most severe disease was developed on *L. perenne* at $35/28^0$ C ($P<0.01$) and the significantly least severe disease was developed on *L. perenne* at $25/18^0$ C ($P<0.01$) **Table 2.3 (a)**, **Figure 2.3 (a, b, c)**.

For *P. annua* that inoculated with *L. perenne* bacterial isolates, r0, r3 and r5, respectively, the significantly most severe disease was developed on *P. annua* at $35/28^0$ C ($P<0.01$) and the significantly least severe disease was developed on *P. annua* at $25/18^0$ C ($P<0.01$) **Table 2.3 (b)**, **Figure 2.3 (d, e, f)**.

Significantly more severe disease was developed on *L. perenne* than *P. annua* at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively, ($P<0.01$, <0.01 and <0.01, respectively) **Table 2.4 (a),** when the pathogens were *L. perenne* bacterial isolates, r0 and r5, respectively. Significantly more severe disease was developed on *L. perenne* than *P. annua* at $25/18^0$ C ($P<0.01$) **Table 2.4**

(a), when the pathogen was *L. perenne* bacterial isolate, r3. No significant difference in disease severity was measured between *L. perenne* and *P. annua* at $30/23^0$ C and $35/28^0$ C, respectively (P>0.01 and >0.01, respectively) **Table 2.4 (a)**, when the pathogen was *L. perenne* bacterial isolate, r3.

At $25/18^0$ C and $30/23^0$ C, the significantly least severe disease was developed on both *L. perenne* and *P. annua*, respectively, when the pathogen was *L. perenne* bacterial isolate, r3, (P<0.01) **Table 2.5 (a)** while no significant difference (P>0.01) in disease severity was measured between hosts (*L. perenne* and *P. annua*, respectively) that were inoculated with *L. perenne* bacterial isolate, r0 **Table 2.5 (a)** and hosts (*L. perenne* and *P. annua*, respectively) that were inoculated with *L. perenne* bacterial isolate, r5 **Table 2.5 (a)**.

At $35/28^0$ C, the significantly most severe disease was developed on *L. perenne* that was inoculated with *L. perenne* bacterial isolate, r5, (P<0.01) **Table 2.5 (a)** and the significantly least severe disease was developed on *L. perenne* that was inoculated with *L. perenne* bacterial isolate, r3 (P<0.01) **Table 2.5 (a)**. At $35/28^0$ C, the significantly least severe disease was developed on *P. annua* that was inoculated with *L. perenne* bacterial isolate, r3, (P<0.01) **Table 2.5 (a)** while no significant difference (P>0.01) in disease severity was measured between *P. annua* that was inoculated with *L. perenne* bacterial isolate, r0 **Table 2.5 (a)** and *P. annua* that was inoculated with *L. perenne* bacterial isolate, r5 **Table 2.5 (a)**.

Temperature influence on disease severity: *Poa annua* and *Lolium perenne* infected with *Poa annua* bacterial isolates (p0, p6 and p8)

For *P. annua* that inoculated with *P. annua* bacterial isolates, p0, p6 and p8, respectively, the significantly most severe disease was developed on *P. annua* at $35/28^0$ C (P<0.01) and the significantly least severe disease was developed on *P. annua* at $25/18^0$ C (P<0.01) **Table 2.3 (b), Figure 2.4 (a, b, c)**.

For *L. perenne* that inoculated with *P. annua* bacterial isolate, p0, the significantly most severe disease was developed on *L. perenne* at $35/28^0$ C ($P<0.01$) and the significantly least severe disease was developed on *L. perenne* at $25/18^0$ C ($P<0.01$) **Table 2.3 (a), Figure 2.4 (d)**. For *L. perenne* that inoculated with *P. annua* bacterial isolates, p6 and p8, respectively, the significantly most severe disease was developed on *P. annua* at $35/28^0$ C ($P<0.01$) while no significantly difference in disease severity was measure between *L. perenne* that was incubated at $25/18^0$ C and *L. perenne* that was incubated at $30/23^0$ C ($P>0.01$) **Table 2.3 (a), Figure 2.4 (e, f)**.

Significantly more severe disease was developed on *P. annua* than *L. perenne* at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively ($P<0.01$, <0.01 and <0.01, respectively) **Table 2.4 (b),** when the pathogens were *P. annua* bacterial isolates, p0, p6 and p8, respectively.

At $35/28^0$ C, significantly most severe disease was developed on *P. annua* that was inoculated with *P. annua* bacterial isolate, p8, ($P<0.01$) **Table 2.5 (b)** and significantly least severe disease was developed on *P. annua* that was inoculated with *P. annua* bacterial isolate, p0 ($P<0.01$) **Table 2.5 (b)**. At $35/28^0$ C, significantly most severe disease was developed on *L. perenne* that inoculated with *P. annua* bacterial isolate, p0, ($P<0.01$) **Table 2.5 (b)** while no significant difference ($P>0.01$) in disease severity was measured between *L. perenne* that was inoculated with *P. annua* bacterial isolate, p6, **Table 2.5 (b)** and *L. perenne* that was inoculated with *P. annua* bacterial isolate, p8 **Table 2.5 (b)**.

At $25/18^0$ C and $30/23^0$ C, no significant difference ($P>0.01$) in disease severity was measured among hosts (*P. annua* and *L. perenne*, respectively) that were inoculated with *P. annua* bacterial isolates, p0, p6 and p8, respectively **Table 2.5 (b)**.

Table 2.3. Difference among disease severity on hosts when they were inoculated with same bacterial isolate but incubated at different temperatures.

(a). *Lolium perenne* inoculated with *Lolium perenne* bacterial isolates, r0, r3 and r5, and *Poa annua* bacterial isolates, p0, p6 and p8, respectively, and incubated at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively.

Hosts	Temperatures	Disease Severity					
		r0	r3	r5	p0	p6	p8
Lolium perenne	25/18℃	0.28(a)	0.14(a)	0.32(a)	0.17(a)	0.19(a)	0.21(a)
	30/23℃	0.34(b)	0.20(b)	0.39(b)	0.25(b)	0.25(a)	0.25(a)
	35/28℃	0.55(c)	0.31(c)	0.62(c)	0.52(c)	0.34(b)	0.39(b)

Disease severities with same little letter in bracket are not significantly different from each other. Significant difference was determined according to Least Significant Difference multiple comparison test ($P<0.01$).

(b). *Poa annua* inoculated with *Poa annua* bacterial isolates, p0, p6 and p8, and *Lolium perenne* bacterial isolates, r0, r3 and r5, respectively and incubated at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively.

Hosts	Temperatures	Disease Severity					
		p0	p6	p8	r0	r3	r5
Poa annua	25/18℃	0.39(a)	0.41(a)	0.41(a)	0.13(a)	0.06(a)	0.13(a)
	30/23℃	0.48(b)	0.52(b)	0.51(b)	0.22(b)	0.17(b)	0.21(b)
	35/28℃	0.61(c)	0.66(c)	0.73(c)	0.45(c)	0.30(c)	0.43(c)

Disease severities with same little letter in bracket are not significantly different from each other. Significant difference was determined according to Least Significant Difference multiple comparison test ($P<0.01$).

Table 2.4. Difference between disease severity on *Lolium perenne* and disease severity on *Poa annua* when both *Lolium perenne* and *Poa annua* were inoculated with the same bacterial isolate and incubated at the same temperature.

(a). *Lolium perenne* and *Poa annua* inoculated with *Lolium perenne* bacterial isolates, r0, r3 and r5, respectively, and incubated at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively.

Temperatures	Hosts	Disease Severity		
		r0	r3	r5
25/18℃	*Lolium perenne*	0.28(a)	0.14(a)	0.32(a)
	Poa annua	0.13(b)	0.06(b)	0.13(b)
30/23℃	*Lolium perenne*	0.34(a)	0.20(a)	0.39(a)
	Poa annua	0.22(b)	0.17(a)	0.21(b)
35/28℃	*Lolium perenne*	0.55(a)	0.31(a)	0.62(a)
	Poa annua	0.45(b)	0.30(a)	0.43(b)

Comparison was conducted between *Lolium perenne* and *Poa annua* but within the same bacterial isolate and the same temperature. Disease severities with same little letter in bracket are not significantly different from each other. Significant difference was determined according to Least Significant Difference multiple comparison test ($P<0.01$).

(b). *Poa annua* and *Lolium perenne* inoculated with *Poa annua* bacterial isolates, p0, p6 and p8, respectively, and incubated at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively.

Temperatures	Hosts	Disease Severity		
		p0	p6	p8
25/18℃	*Poa annua*	0.39(a)	0.41(a)	0.41(a)
	Lolium perenne	0.17(b)	0.19(b)	0.21(b)
30/23℃	*Poa annua*	0.48(a)	0.52(a)	0.51(a)
	Lolium perenne	0.25(b)	0.25(b)	0.25(b)
35/28℃	*Poa annua*	0.61(a)	0.66(a)	0.73(a)
	Lolium perenne	0.52(b)	0.34(b)	0.39(b)

Comparison was conducted between *Poa annua* and *Lolium perenne* but within the same bacterial isolate and the same temperature. Disease severities with same little letter in bracket are not significantly different from each other. Significant difference was determined according to Least Significant Difference multiple comparison test ($P<0.01$).

Table 2.5. Difference among disease severity on the same host (*Lolium perenne* and *Poa annua*, respectively) that was inoculated with different bacterial isolates but incubated at the same temperature.

(a). *Lolium perenne* and *Poa annua* inoculated with *Lolium perenne* bacterial isolates, r0, r3 and r5, respectively, and incubated at 25/18^0 C, 30/23^0 C and 35/28^0 C, respectively.

Hosts	Isolates	Disease Severity		
		25/18℃	30/23℃	35/28℃
Lolium perenne	r0	0.28(a)	0.34(a)	0.55(a)
	r3	0.14(b)	0.20(b)	0.31(b)
	r5	0.32(a)	0.39(a)	0.62(c)
Poa annua	r0	0.13(a)	0.22(a)	0.45(a)
	r3	0.06(b)	0.17(b)	0.30(b)
	r5	0.13(a)	0.21(a)	0.43(a)

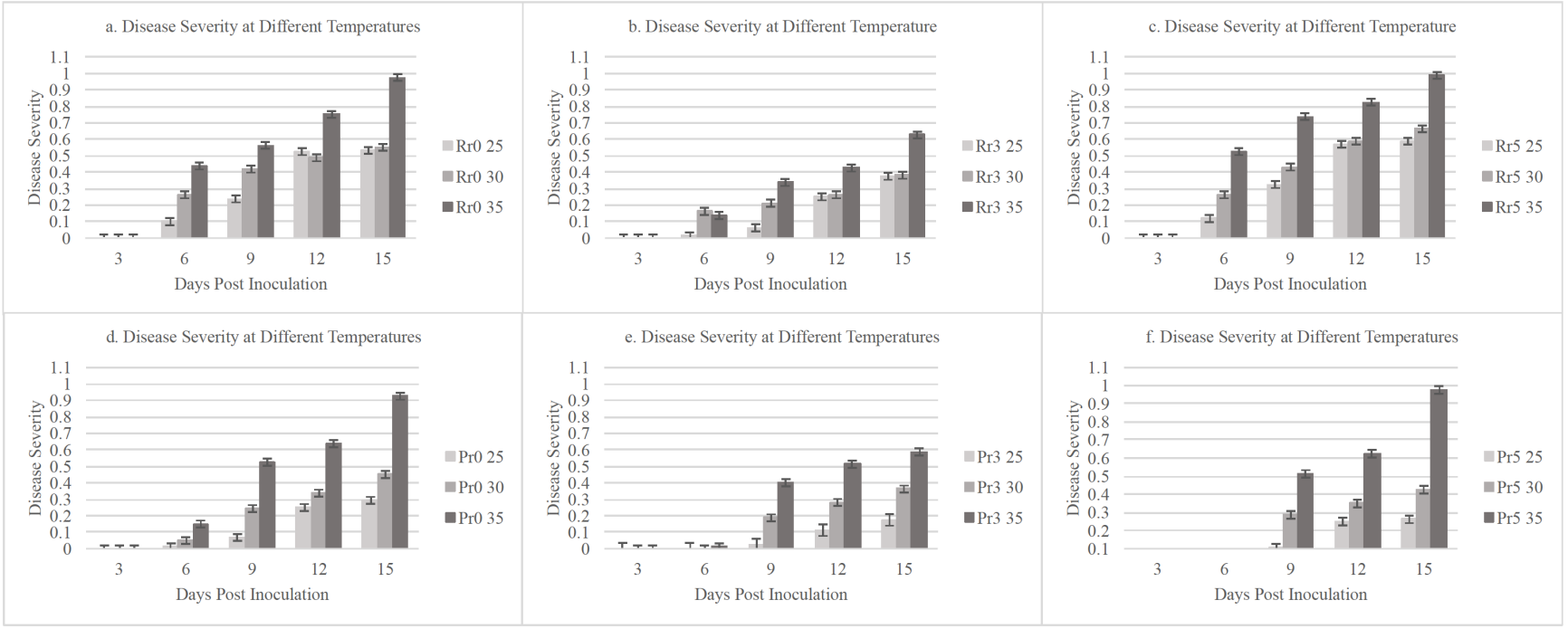
Pairwise comparison was conducted among different bacterial isolates but within the same host and the same temperature. Disease severities with same little letter in bracket are not significantly different from each other. Significant difference was determined according to Least Significant Difference multiple comparison test (P<0.01).

(b). *Poa annua* and *Lolium perenne* inoculated with *Poa annua* bacterial isolates, p0, p6 and p8, respectively, and incubated at 25/18^0 C, 30/23^0 C and 35/28^0 C, respectively.

Hosts	Isolates	Disease Severity		
		25/18℃	30/23℃	35/28℃
Poa annua	p0	0.39(a)	0.48(a)	0.61(a)
	p6	0.41(a)	0.52(a)	0.66(ab)
	p8	0.41(a)	0.51(a)	0.73(b)
Lolium perenne	p0	0.17(a)	0.25(a)	0.52(a)
	p6	0.19(a)	0.25(a)	0.34(b)
	p8	0.21(a)	0.25(a)	0.39(b)

Pairwise comparison was conducted among different bacterial isolates but within the same host and the same temperature. Disease severities with same little letter in bracket are not significantly different from each other. Significant difference was determined according to Least Significant Difference multiple comparison test (P<0.01).

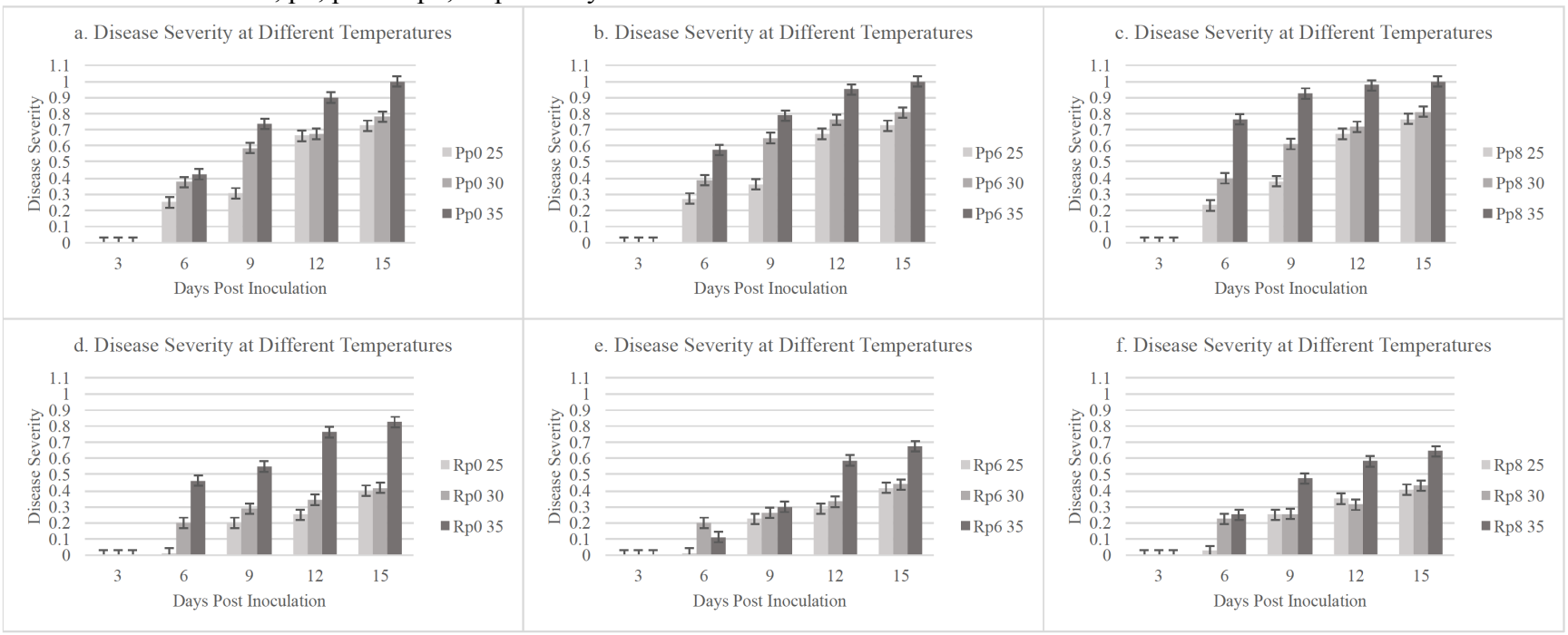
Figure 2.3 (a-f). Temperature influence on disease severity on *Lolium perenne* (R) and *Poa annua* (P) that were inoculated with *Lolium perenne* bacterial isolates, r0, r3 and r5, respectively.



Error bars represent standard error of mean (n=4).

Rr0, Rr3 and Rr5 represent *Lolium perenne* inoculated with *Lolium perenne* bacterial isolates r0, r3 and r5, respectively. Pr0, Pr3 and Pr5, represent *Poa annua* inoculated with *Lolium perenne* bacterial isolates r0, r3 and r5, respectively.

Figure 2.4 (a-f). Temperature influence on disease severity on *Poa annua* (P) and *Lolium perenne* (R) that were inoculated with *Poa annua* bacterial isolates, p0, p6 and p8, respectively.



Error bars represent standard error of mean (n=4).

Pp0, Pp6 and Pp8represent *Poa annua* inoculated with *Poa annua* bacterial isolates p0, p6 and p8, respectively. Rp0, Rp6 and Rp8, represent *Lolium perenne* inoculated with *Poa annua* bacterial isolates p0, p6 and p8, respectively.

DISSCUSSION

The leaves clipping-inoculation of the three *L. perenne* bacterial isolates and the three *P. annua* bacterial isolates on *L. perenne* and *P. annua* at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively, caused significant disease on *L. perenne* and *P. annua* at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively, when compared to PBS control. This result is consistent with the mode of transmission and spread of *X. translucens pv. graminis* on *L. perenne* [7, 8] and *X. translucens pv. poae* on *P. annua* [4, 5, 6]. And the mode of transmission and spread is bacterial wilt can be easily induced via wounds on leaves.

Egli et al. [9] and Egli and Schmidt [10] specified that *X. translucens pv. graminis* from *L. multiflorum* was pathogenic to both *L. perenne* and *P. trivialis* and specified that *X. translucens pv. poae* from *P. trivialis* was pathogenic to both *P. annua* and *L. multiflorum*. Our study indicates that the *L. perenne* bacterial isolates were pathogenic to both *L. perenne* and *P. annua* and indicates that the *P. annua* bacterial isolates were pathogenic to both *P. annua* and *L. perenne*. Our study reveals that *L. perenne* was significantly more susceptible to *L. perenne* bacterial isolates, r0 and r5, than *P. annua* bacterial isolates $25/18^0$ C, $30/23^0$ C, respectively, **Table 2.2 (b)** and reveals that *P. annua* was more susceptible to *P. annua* bacterial isolates than *L. perenne* bacterial isolates at $25/18^0$ C, $30/23^0$ C and $35/28^0$ C, respectively **Table 2.2 (b)**. These results that bacterial isolates were more pathogenic to the hosts from which they were isolated are consistent with previous study [9, 10]. There is an exception that *L. perenne* was as susceptible to p0 as to r0 and r5 at $35/28^0$ C **Table 2.2 (b)**. Our study demonstrated that *L. perenne* was significantly more susceptible to the high temperature, $35/28^0$ C, than *P. annua*. Our study also demonstrated that the in-vitro generation times of p0, r0 and r5 are the significantly shortest at $35/28^0$ C **Table 2.1 (a)** and were not significantly different from each other **Table 2.1**

(b). These results indicate that *L. perenne* suffered heavy stress from both temperature and bacteria at $35/28^0$ C so that was succumbed to high-temperature induced physiological decline **Figure 2.2 (c)**. It may be reason of the exception.

Our study indicates that the optimal growth temperature for *P. annua* bacterial isolates in liquid culture (XBM) is 35^0 C **Table 2.1 (a)**, which is consistent with previous study [4, 11] that the optimal growth temperature of liquid culture for *X. translucens pv. poae* from *P. annua* is between 30^0 C and 35^0 C.

Our study indicates the in-vitro generation time of *P. annua* bacterial isolates is significantly shortest at 35^0 C while there is no significantly difference between in-vitro generation time of *P. annua* bacterial isolates at 25^0 C and in-vitro generation time of *P. annua* bacterial isolates at 30^0 C **Table 2.1 (a)**. Our study also indicates that the optimal growth temperature for *L. perenne* bacterial isolates in liquid culture (XBM) is 35^0 C and indicates that the in-vitro generation time of *L. perenne* bacterial isolates is significantly shortest at 35^0 C while there is no significantly difference between in-vitro generation time of *L. perenne* bacterial isolates at 25^0 C and in-vitro generation time of *L. perenne* bacterial isolates at 30^0 C **Table 2.1 (a)**. These results fill the gap that few studies were conducted to investigate the in-vitro generation time of bacterial wilt bacteria from *P. annua,* the in-vitro optimal growth temperature and in-vitro generation time of bacterial wilt bacteria from *L. perenne*.

Our study indicates that significant disease was firstly developed on *L. perenne* and *P. annua* 6 dpi at $25/18^0$ C, $30/23^0$ C and $35/18^0$ C, respectively, when *L. perenne* was inoculated with *L. perenne* bacterial isolate, r0 and r5, respectively, **Table 2.2 (a)** and when *P. annua* was inoculated with *P. annua* bacterial isolates, p0, p6 and p8, respectively **Table 2.2 (a)**. Our study also revealed that there was a delay in significant disease development when *L. perenne* was

inoculated with *P. annua* bacterial isolates at $25/18^0$ C **Table 2.2 (a)** and when *P. annua* was inoculated with *L. perenne* bacterial isolates, r0 and r5, at $25/18^0$ C, $30/23^0$ C and $35/18^0$ C, respectively **Table 2.2 (a)**. The delay in disease development may be due to the host resistance to pathogen from different origin.

Our study demonstrated positive correlation between disease severity and temperature which is consistent with previous study [11, 12, 13, 14, 15] and specified the most severe disease developed at $35/18^0$ C while the least severe disease developed at $25/18^0$ C. But there is one exception that there was no significant difference in disease severity between $25/18^0$ C and $30/23^0$ C when *L. perenne* was inoculated with *P. annua* bacterial isolates, p6 and p8, respectively **Table 2.3 (a)**. The exception may be due to the host resistance to pathogens that come from different origin.

Our study revealed that temperature is main factor that made significant difference in disease severity between $25/18^0$ C and $30/23^0$ C. Because our study revealed that there was no significant difference in in-vitro generation time between $25/18^0$ C and $30/23^0$ C for each of the bacterial isolates used in this study **Table 2.1 (a)** and our study also revealed that the wilt symptom on hosts was significantly more obvious at $30/23^0$ C than $25/18^0$ C. Our study revealed that it was the combination of the stresses from both temperature and bacteria that made the most severe disease developed at $35/28^0$ C. Because our study revealed that not only the wilt symptom on hosts was significantly more obvious at $35/28^0$ C than $30/23^0$ C but also the in-vitro generation time was the significantly shortest at $35/28^0$ C for all the bacterial isolates used in this study.

Our study revealed that *L. perenne* bacterial isolates, r0 and r5, were more pathogenic to *L. perenne* than *P. annua* at $25/18^0$ C, $30/23^0$ C and $35/18^0$ C, respectively, and revealed that *P. annua* bacterial isolates were more pathogenic to *P. annua* than to *L. perenne* at $25/18^0$ C, $30/23^0$

C and $35/18^0$ C, respectively, which is consistent with result that bacterial isolates are more pathogenic to the hosts from which they are isolated in previous study [9, 10].

Our study revealed that the varieties in virulence among bacterial isolates at $35/28^0$ C were not consistent with that at $25/18^0$ C and $30/23^0$ C. Our data cannot explain the inconsistence. The inconsistence may be due to the combined function of host resistance, high-temperature influence and bacterial virulence gene.

L. perenne bacterial isolate, r3, is an exception. Comparing to *L. perenne* bacterial isolates, r0 and r5, there was a delay in disease development when *L. perenne* was inoculate with *L. perenne* bacterial isolate, r3, at $25/18^0$ C and $35/18^0$ C, respectively, **Table 2.2 (a)** and the disease severity caused by r3 on *L. perenne* 15 dpi was not significantly different from PBS control 15dpi at $30/23^0$ C **Table 2.2 (a)**. What's more, comparing to *P. annua* bacterial isolates, *L. perenne* bacterial isolate, r3, was not significantly more pathogenic to *L. perenne*. These results indicate that *L. perenne* bacterial isolate, r3, is different from all other five bacterial isolates (r0, r5, p0, p6 and p8).

This study elucidated the host range, the in-vitro generation time and the in-vitro optimal growth temperature of the six bacterial isolates and elucidated optimal air temperature for the six bacterial isolates causing most severe disease on hosts and the pathogenicity and virulence of the six bacterial isolates. Most results are consistent with previous study [9, 10] which suggest that the *L. perenne* bacterial isolates, r0 and r5, are *X. translucens pv. graminis* while *P. annua* bacterial isolates are *X. translucens pv. poae.* Depending on the dramatically different properties of *L. perenne* bacterial isolate, r3, it suggests that *L. perenne* bacterial isolate, r3, belongs to a species other than *X. translucens pv. graminis* and *X. translucens pv. poae*. Further research is required to identify those bacterial isolates.

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LITERATURE CITED

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CHAPTER THREE

IDENTIFICATION OF BACTERIA ISOLATED FROM *LOLIUM PERENNE* AND *POA ANNUA*

ABSTRACT

Three *Lolium perenne* bacterial isolates (r0, r3 and r5) and three *Poa annua* bacterial isolates (p0, p6 and p8), which could infect both *L. perenne* and *P. annua*, were used in this study. A partial 16S rDNA sequence analysis of *Stenotrophomonas maltophilia*, the 6 bacterial isolates and other 13 reference *Xanthomonas* species (15 strains) was conducted using a 1276-bp 16S rDNA sequence, a total of 22 sequences, which assigned *P. annua* bacterial isolates, p0, p6 and p8 to *Xanthomonas translucens*, assigned *L. perenne* bacterial isolates, r0 and r5, to *Xanthomonas translucens pv. graminis* while failed to confirm the identity of *L. perenne* bacterial isolate, r3, at species level. A multilocus sequence analysis (MLSA) of *S. maltophilia*, the 6 bacterial isolates and other 41 reference *Xanthomonas* species (60 strains) was conducted using a 1803-bp concatenated sequence of four housekeeping genes, *gyrB*, *fusA*, *gapA* and *lepA*, a total of 67 sequences, which resulted in two distinct groups and assigned bacterial isolates, r0, r5, p0, p6 and p8, to one group while assigned bacterial isolate, r3, to another group. The MLSA also revealed *P. annua* bacterial isolates, p0, p6 and p8, cluster with *Xanthomonas translucens pv. poae*, *L. perenne* bacterial isolates, r0 and r5, cluster with *Xanthomonas translucens pv. graminis* while *L. perenne* bacterial isolate, r3, is in an independent clade. The distance matrix indicated *L. perenne* bacterial isolate, r3, has the closest relationship to *Xanthomonas vasicola*. Based on the data produced, it indicates that the three *P. annua* bacterial isolates are *X. translucens pv. poae*, the *L. perenne* bacterial isolates, r0 and r5, are *X. translucens pv. graminis* while *L. perenne* bacterial isolate, r3, may be a new *Xanthomonas* species. The research also indicates that MLSA offers a relatively

simple way of identifying strains as members of known species, especially, infraspecies, or indicating their status as members of new species.

INTRODUCTION

Bacterial wilt was firstly described in 1975 attacking breeding lines of *Lolium* spp., *Dactylis* spp. and *Festuca* spp. in Switzerland [1]. The causal agent of this disease was firstly named *Xanthomonas graminis* by Egli et al. [2] and this name was changed to *Xanthomonas campestris pv. graminis* by Dye et al. later [3]. Subsequently, the causal agent of bacterial wilt of *P. annua* was identified as *Xanthomonas campestris pv. poae* [4]. More recently, *X. campestris pv. graminis* and *X. campestris pv. poae* were reclassified to *Xanthomonas translucens* pathovars [5].

Channon et al. [1] indicated that *L. perenne* was either moderately or highly susceptible to *X. translucens pv. graminis* (*X. t. g*) (uncertain biological origin). Egli et al. [4] indicated that *L. perenne* was highly susceptible to *X. t. g* (*Lolium multiflorum* bacterial isolates) but largely resistant to *X. translucens pv. poae* (*X. t. poae*) (*Poa trivialis* bacterial isolates). Leyns et al. [6] indicated that *L. perenne* could be readily infected with *X. t. g* (*L. perenne* bacterial isolates) with obvious disease expression. Kölliker et al. [7] indicated that the *X. t. g* was either moderately or highly pathogenic to *L. multiflorum* and described the evolutionary relationship among *X. t. g* (*L. multiflorum* isolates), other *Xanthomonas translucens* spp. and few other *Xanthomonas* spp. based on 16S rDNA sequences. Bacterial wilt of *L. perenne* was firstly reported in 2015 in the United States [8]. It was demonstrated that the causal agent of the newly discovered disease could infect both *L. perenne* and *P. annua*. Though a lot of studies [1, 4, 6, 7] about the host-parasite interaction between forage grass (*Lolium* spp., *Dactylis* spp. and *Festuca* spp.) and *X. t. g* (mostly *Lolium multiflorum* bacterial isolates) have been done, there was only a few studies about the *L. perenne* bacterial isolates that could cause bacterial wilt on both *L. perenne* and *P. annua*. What's more,

most of the previous studies were restricted in Europe while bacterial wilt of *L. perenne* is a newly discovered disease in the United states, so, it is necessary to confirm identity of the causal agent of the newly discovered disease and illustrate the evolutionary relationship of the causal agent to other *Xanthomonas* spp.

Egli et al. [4] indicated that *P. annua* was intermediately susceptible to *X. t. poae* (*P. trivialis* bacterial isolates) but largely resistant to *X. t. g* (*L. multiflorum* bacterial isolates). Bacterial wilt of *P. annua* was first reported in 1984 in the United States [9]. Mitkowski et al. [10] demonstrated that *X. t. poae* is the causal agent of bacterial wilt of *P. annua* and Mitkowski et al. [11] also described the evolutionary relationship among *X. t. poae* (*P. annua* bacterial isolates) and other *Xanthomonas translucens* spp. based on the partial 16S rDNA sequences, the complete intergenic spacer region sequences and the partial 23S rDNA sequences. However, there is still a lack of knowledge about the identity of the *P. annua* bacterial isolates that could cause bacterial wilt on both *P. annua* and *L. perenne* and there is still a lack of knowledge about the evolutionary relationship among the *P. annua* bacterial isolate and other *Xanthomonas* spp.

Young et al. [18] and Almeida et al. [19] described the evolutionary relationship among the strains in genus *Xanthomonas* based on the concatenated sequences of several housekeeping genes. However, *Xanthomonas translucens* spp. were not discussed in detail in Young et al. [18] while *Xanthomonas translucens* spp. were not included in Almeida et al. [19].

In previous study, it was demonstrated that both the *L. perenne* bacterial isolates and the *P. annua* bacterial isolates could infect both *L. perenne* and *P. annua* but they were more pathogenic to the host from which they were isolated. In this study, the *L. perenne* bacterial isolates and the *P. annua* isolates were designated as the putative *X. t. g* isolates and the putative *X. t. poae* isolates, respectively. Analysis of partial sequence of 16S rDNA was conducted to confirm the species

identities of the putative *X. t. g* isolates and the putative *X. t. poae* isolates. Multilocus analysis of concatenated sequence of four housekeeping genes was conducted to confirm the infraspecies identities of the putative *X. t. g* isolates and the putative *X. t. poae* isolates and describe the evolutionary relationship among them and other 41 *Xanthomonas* spp.

MATERIALS AND METHODS

Strains

The three putative *X. t. g* isolates (r0, r3 and r5) and the three putative *X. t. poae* isolates (p0, p6 and p8) were used in both analysis of 16S rDNA sequence and analysis of multilocus sequence. Reference strains and sequences for analysis of 16S rDNA sequences were obtained from the National Center for Biotechnology Information (GenBank) database **Table 3.1**. Reference strains and sequences for analysis of multilocus sequence were obtained from both GenBank and Plant Associated and Environmental Microbe (PAMDB) database **Table 3.2**.

Table 3.1. Strains used in analysis of 16S rDNA sequences.

Strains	Abbreviation of names	Database	GenBank accession numbers
S. maltophilia(T) LMG958	*S.maltophilia(T)*	GenBank	X95923.1
X. cassavae(T) LMG673	*X.cassavae(T)*	GenBank	Y10762.1
X. bromi(T) LMG947	*X.bromi(T)*	GenBank	Y10764.1
X. oryzae pv. oryzae(T) LMG5047	*X.o.o(T)*	GenBank	X95921.1
X. campestris pv. campestris(T) LMG568	*X.cam.cam(T)*	GenBank	X95917.1
X. sacchari(T) LMG471	*X.sacchari(T)*	GenBank	Y10766.1
X. albilineans(T) LMG494	*X.albilineans(T)*	GenBank	X95918
X. hyacinthi(T) LMG739	*X.hyacinthi(T)*	GenBank	Y10754
X. melonis(T) LMG8670	*X.melonis(T)*	GenBank	Y10756
X. translucens pv. arrhenatheri(T) LMG727	*X.t.arr(T)*	Genbank	AY855844.1
X. translucens pv. translucens(T) DSM18974 isolate peng1	*X.t.t(T)*	GenBank	NZ_LT604072.1
X. translucens pv. undulosa Xtu4699	*X.t.undu(T)*	GenBank	CP008714.1
X. translucens pv. poae(T) LMG728	*X.t.poae(T)*	GenBank	AY855845.1
X. translucens pv. poae(1) ATCC33804	*X.t.poae(1)*	GenBank	AY572961
X. translucens pv. graminis(T) LMG726	*X.t.g(T)*	GenBank	X99298
X. translucens pv. graminis(1) CFBP2053	*X.t.g(1)*	GenBank	NZ_LHSI01000001.1
r0 (*X. translucens pv. graminis* putative isolate)	*r0*	N/A	N/A
r3 (*X. translucens pv. graminis* putative isolate)	*r3*	N/A	N/A
r5 (*X. translucens pv. graminis* putative isolate)	*r5*	N/A	N/A
p0 (*X. translucens pv. poae* putative isolate)	*p0*	N/A	N/A
p6 (*X. translucens pv. poae* putative isolate)	*p6*	N/A	N/A
p8 (*X. translucens pv. poae* putative isolate)	*p8*	N/A	N/A

The table includes three putative *X. t. g* isolates (r0, r3 and r5), three putative *X. t. poae* isolates (p0, p6 and p8), 15 reference *Xanthomonas* strains and the reference outgroup strain, *S. maltophilia* (*Stenotrophomonas maltophilia*). The "(T)" indicates the type strain. The "(1)" indicates the strain other than the type strain.

Table 3.2. Strains used in analysis of multilocus sequences.

Strains	Names(abbreviation)	Databases	GenBank accession numbers
*X. alfalfae pv. alfalfae*** ATCC11765 (112)	*X.alf.alf***	PAMDB	N/A
*X. campestris pv. armoraciae*** 756C (115)	*X.c.arm***	PAMDB	N/A
*X. fuscans pv. aurantifolii*** pathotype B 1622 (116)	*X.fus.aur**(1)*	PAMDB	N/A
*X. fuscans pv. aurantifolii*** pathotype C ATCC51302 (117)	*X.fus.aur**(2)*	PAMDB	N/A
*X. axonopodis pv. begonia*** LMG 7189 (120)	*X.axo.beg***	PAMDB	N/A
*X. campestris*** ATCC31313 (128)	*X.cam**(1)*	PAMDB	N/A
*X. campestris*** ATCC43304 (130)	*X.cam**(2)*	PAMDB	N/A
*X. campestris*** ATCC31600 (133)	*X.cam**(3)*	PAMDB	N/A
*X. citri pv. citri*** ATCC49119 (152)	*X.cit.cit***	PAMDB	N/A
*X. citri*** ICPB10409 (156)	*X.cit***	PAMDB	N/A
*X. alfalfae pv. citrumelonis*** 1887 CBS (158)	*X.alf.citru**(1)*	PAMDB	N/A
*X. alfalfae pv. citrumelonis*** 1902 CBS (159)	*X.alf.citru**(2)*	PAMDB	N/A
*X. alfalfae pv. citrumelonis*** LMG 9325 (160)	*X.alf.citru**(3)*	PAMDB	N/A
*X. euvesicatoria*** ATCC11633 (163)	*X.euv***	PAMDB	N/A
*X. fuscans pv. fuscans*** ATCC13464 (166)	*X.fus.fus***	PAMDB	N/A
*X. axonopodis pv. vignicola*** LMG8752 (199)	*X.axo.vig***	PAMDB	N/A
*X. axonopodis pv. glycines*** ATCC11766 (169)	*X.axo.gly(1)***	PAMDB	N/A
*X. hortorum pv. hederae*** LMG733 (170)	*X.hor.hed***	PAMDB	N/A
*X. maculifoliigardeniae*** BAV37 (171)	*X.mac***	PAMDB	N/A
*X. citri pv. malvacearum*** ATCC12131 (177)	*X.cit.mal(1)***	PAMDB	N/A
*X. citri pv. malvacearum*** ATCC49290 (178)	*X.cit.mal(2)***	PAMDB	N/A
*X. citri pv. malvacearum*** ATCC14982 (179)	*X.cit.mal(3)***	PAMDB	N/A
*X. axonopodis pv. manihotis*** LMG 784 (180)	*X.axo.man(1)***	PAMDB	N/A
*X. axonopodis pv. manihotis*** ATCC23380 (181)	*X.axo.man(2)***	PAMDB	N/A
*X. vasicola*** LMG785 (182)	*X.vas***	PAMDB	N/A
*X. nigromaculans*** LMG787 (183)	*X.nig***	PAMDB	N/A
*X. oryzae pv. oryzicola*** BLS256 (184)	*X.ory.oryzi***	PAMDB	N/A
*X. oryzae pv. oryzae*** DX-027 (1591)	*X.ory.ory(1)***	PAMDB	N/A
*X. axonopodis pv. phaseoli*** ICPB10532 (188)	*X.axo.pha(1)***	PAMDB	N/A
*X. axonopodis pv. phaseoli*** ATCC49119 (191)	*X.axo.pha(2)***	PAMDB	N/A
*X. pisi*** LMG847 (194)	*X.pis***	PAMDB	N/A
*X. vesicatoria*** ATCC11551 (196)	*X.ves(1)***	PAMDB	N/A
*X. vesicatoria*** ICMP63 (664)	*X.ves(2)***	PAMDB	N/A
*X. maltophilia*** 66 R551 3 (375)	*X.mal***	PAMDB	N/A
*X. fastidiosa*** 66 9a5c (376)	*X.fas***	PAMDB	N/A
*X. axonopodis pv. axonopodis*** ICMP50 (662)	*X.axo.axo(1)***	PAMDB	N/A
*X. axonopodis pv. axonopodis*** ICMP698 (687)	*X.axo.axo(2)***	PAMDB	N/A
*X. hortorum pv. taraxaci*** ICMP579 (684)	*X.hor.tar***	PAMDB	N/A
*X. arboricola pv. corylina*** ICMP5726 (708)	*X.arb.cor***	PAMDB	N/A
*X. axonopodis pv. glycines*** ICMP5732 (709)	*X.axo.gly(2)***	PAMDB	N/A
*X. bromi*** ICMP12545 (760)	*X.bromi(1)***	PAMDB	N/A
*X. gardneri*** ICMP16689 (766)	*X.gar***	PAMDB	N/A
*X. axonopodis pv. poinsettiicola*** (948)	*X.axo.poi***	PAMDB	N/A
*X. oryzae pv. oryzae*** ANBB2 (1563)	*X.ory.ory(2)***	PAMDB	N/A
*X. oryzae pv. oryzae*** DX-020 (1590)	*X.ory.ory(3)***	PAMDB	N/A
S. maltophilia(T) LMG958	*S.maltophilia(T)*	GenBank	CP008838.1(gyrB, fusA, gapA and lepA)
X. cassavae(T) LMG673	*X.cassavae(T)*	GenBank	HM569156.1(gyrB), NZ_CM002139.1(fusA), NZ_CM002139.1(gapA), KF306175.1(lepA).
X. bromi(T) LMG947	*X.bromi(T)*	GenBank	HM569149.1(gyrB), NZ_FLTX01000004.1(fusA), FLTX01000054.1(gapA), FLTX01000040.1(lepA).
X. campestris pv.campestris(T) LMG568	*X.c.c(T)*	GenBank	NC_003902.1(gyrB, fusA, gapA and lepA)
*X. sacchari** R1	*X.sacchari(1)**	GenBank	CP010409.1(gyrB, fusA, gapA and lepA)
*X. sacchari** LMG 476	*X.sacchari(2)**	GenBank	NZ_JXQE01000055.1 (gyrB), NZ_JXQE01000055.1 (fusA), NZ_JXQE01000055.1 (gapA), NZ_JXQE01000055.1 (lepA).
*X. albilineans** GPE PC86	*X.albilineans(1)**	GenBank	NZ_JZIB01000003.1(gyrB, fusA, gapA and lepA)
*X. albilineans** GPE PC73	*X.albilineans(2)**	GenBank	FP565176.1(gyrB, fusA, gapA and lepA)
X. hyacinthi(T) LMG739	*X.hyacinthi(T)*	GenBank	HM569190.1(gyrB), JPLD01000246.1(fusA), JPLD01000358.1(gapA), KF306177.1(lepA).
X. translucens pv.arrhenatheri(T) LMG727	*X.t.arr(T)*	Genbank	CXOI01000055.1(gyrB), CXOI01000080.1(fusA), CXOI01000033.1(gapA),CXOI01000026.1(lepA).
X. translucens pv.translucens(T) DSM18974 isolate peng1	*X.t.t(T)*	GenBank	LT604072.1(gyrB, fusA, gapA and lepA)
X. translucens pv. undulosa Xtu4699	*X.t.undu(T)*	GenBank	CP008714.1(gyrB, fusA, gapA and lepA)
X. translucens pv. poae(T) LMG728	*X.t.poae(T)*	GenBank	NZ_CXOK01000082.1 (gyrB), NZ_CXOK01000058.1 (fusA), CXOK01000010.1(gapA), CXOK01000064.1(lepA).
X. translucens pv. poae(1) ATCC33804	*X.t.poae(1)*	GenBank	NZ_MADN01000240.1 (gyrB), NZ_MADN01000218.1 (fusA) NZ_MADN01000199.1 (gapA), NZ_MADN01000253.1 (lepA).
X. translucens pv. graminis(T) LMG726	*X.t.g(T)*	GenBank	NZ_LHSI01000001.1(gyrB, fusA, gapA and lepA)
X. translucens pv. graminis(1) CFBP2053	*X.t.g(1)*	GenBank	NZ_LHSI01000001.1(gyrB, fusA, gapA and lepA)
r0 (X. translucens pv. graminis putative isolate)	*r0*	N/A	N/A
r3 (X. translucens pv. graminis putative isolate)	*r3*	N/A	N/A
r5 (X. translucens pv. graminis putative isolate)	*r5*	N/A	N/A
p0 (X. translucens pv. poae putative isolate)	*p0*	N/A	N/A
p6 (X. translucens pv. poae putative isolate)	*p6*	N/A	N/A
p8 (X. translucens pv. poae putative isolate)	*p8*	N/A	N/A

The table includes the three putative *X. t. g* isolates (r0, r3 and r5), the three putative *X. t. poae* isolates (p0, p6 and p8), 60 reference *Xanthomonas* strains and the reference outgroup strain, *S. maltophilia* (*Stenotrophomonas maltophilia*). "*" indicates the strain is not the same strain that is used in the analysis of 16S rDNA sequences. "**" indicates the strain is obtained from PAMDB. The "(T)" indicates the type strain. The "(1)" indicates the strain other than the type strain.

DNA extraction

Bacteria were streaked on NA and incubated at 23^0 C for 5 days. A single colony was grown in 50 mL *Xanthomonas* broth media (XBM) (1L distilled water, 0.5g K_2HPO_4, 0.5g $NH_4H_2PO_4$, 0.2g $MgSO_4 \bullet H_2O$, 5g NaCl, 10g Yeast Extract and 10g Sucrose) for 48 h at 23^0 C and 1 mL cell suspension was centrifuged at 12400 rpm (Micro Centrifuge model 235C (Fisher Scientific, Hampton, NH, US)) for 3 min. Genomic DNA was extracted from the pellet using a DNeasy® Blood & Tissue Kit (50) (QIAGENE, Hilden, North Rhine-Westphalia, Germany) according to the manufacturer's instructions. DNA concentration was measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, US) and standardized to 10 ng/µL.

Analysis of 16S rDNA sequences and multilocus sequences

Polymerase chain reaction (PCR) amplification of 16S rDNA sequences of the three putative *X. t. g* isolates and the three putative *X. t. poae* isolates were accomplished using two primer sets **Table 3.3**. The first primer set, XAN1(F)/XAN2(R) [12], was used to amplify and sequence the outer1000 bp of the sequence. The second primer set, XAN3(F)/XAN4(R) [10], was used to amplify and sequence the next 1000 bp (derived from the purified PCR products of XAN1(F)/XAN2(R) amplification).

The primer sets **Table 3.3** for PCR amplification and sequencing of the four housekeeping genes, *dnaK, fyuA, gyrB1* and *rpoD*, were obtained from Young et al. [18] and the primer sets **Table 3.3** for amplification and sequencing of the six housekeeping genes, *fusA, gapA, gltA, gyrB, lacF* and *lepA*, were obtained from Almeida et al. [19].

PCR amplifications were performed in an Applied Biosystems 2720 Thermal Cycler (Thermo Scientific, Waltham, MA, US). Each PCR reaction tube were consisted of 12.5 µL Dream Taq Green PCR Master Mix (2X) (Thermo Scientific, Waltham, MA, US), 6.5 µL distilled water, 2.5

μL upstream primer (0.1 nM/μL), 2.5 μL downstream primer (0.1 nM/μL) and 1μL DNA template (10 ng/μL). The procedures for application of PCR amplifications were listed in **Table 3.4**.

PCR products were purified using a QIAquick® PCR Purification Kit (50) (QIAGENE, Hilden, North Rhine-Westphalia, Germany) according to the manufacturer's instructions. Concentrations of the purified PCR products were measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Waltham, MA, US). Purified PCR products were sequenced using an ABI 3730 automated sequencer at the Michigan State University Research Technology Support Facility (East Lansing, MI, US). Sequences were read using Finch TV (Version 1.5.0).

Sequences were aligned with MUSCLE embedded in MEGA7 using default parameters, then both sides of each alignment were trimmed to the sizes determined by reference sequences and trimmed sequences were concatenated to contiguous sequences. Variable loci among multi-aligned sequences were marked using MEGA7. Pair-wise distance matrixes of multi-aligned sequences were constructed using MEGA7 with a 1000-replicate bootstrap method for variance estimation and a p-distance substitution model. Concatenated sequences were compared using MEGA7 with neighbor-joining (NJ) algorithm. For the NJ analysis, a p-distance substitution model was used. Support nodes were based on 1000 bootstrap replicates.

Table 3.3. Primers for amplification and sequencing of 16S rDNA genes and housekeeping genes.

Primers	Sequences
XAN1	F: GGAGAGTTAGATCTTGGCTCAG
XAN2	R: CCGGGTTTCCCCATTCGG
XAN3	F: CCCGGTGTAGCAGTGAAATG
XAN4	R: ATGCAGGTTACTTGTGAGGACG
dnaK	F: GGTGGAAGACCTGGTCAAGA
	R: TCCTTGACYTCGGTGAACTC
fyuA	F: AGCTACGAYGTGCGYTACGA
	R: GTTCACGCCRAACTGGTAG
gyrB1	F: ACGAGTACAACCCGGACAA
	R: CCCATCARGGTGCTGAAGAT
rpoD	F: TGGAACAGGGCTATCTGACC
	R: CATTCYAGGTTGGTCTGRTT
fusA	F: TCTGGCSCARGARGAYCC
	R: GCCTCTTCGTARTGGTCRAA
gapA	F: GGCAATCAAGGTTGGYATCAACG
	R: ATCTCCAGGCACTTGTTSGARTAG
gltA	F: ATCTTGATCAGGTCACGCTCAAC
	R: AGCATCTTCAGCACGGCTTCGTT
gyrB	F: AAGTTCGACGACAACAGCTACAA
	R: GAMAGCACYGCGATCATGCCTTC
lacF	F: SAGRTTCCACCACTTGAAGC
	R: SAGRTTCCACCACTTGAAGC
lepA	F: AAGCSCAGGTGCTCGACTCCAAC
	R: CGTTCCTGCACGATTTCCATGTG

Table 3.4. Procedures of PCR amplification.

	Initial denaturation	Denaturation	Annealing	Extention	Final extention
16S rDNA	95℃, 3min	95℃, 30s, 30 cycles	53℃, 30s, 30 cycles	72℃, 2min, 30 cycles	72℃, 10min
dnaK, fyuA, gyrB1, rpoD	95℃, 3min	95℃, 30s, 30 cycles	52℃, 30s, 30 cycles	72℃, 2min, 30 cycles	72℃, 10min
fusA, gapA, gltA, gyrB, lacF, lepA	95℃, 3min	95℃, 30s, 30 cycles	53℃, 30s, 30 cycles	72℃, 2min, 30 cycles	72℃, 10min

RESULTS

Analysis of 16S rDNA sequences

Partial sequences of 16S rDNA were multi-aligned with MUSCLE [13] embedded in MEGA7 [14] using default parameters and both ends of each alignment were trimmed and the trimmed 16S rDNA sequences were concatenated to give a total length of 1276 bp (positions 174-1449, *X. t. g(T)* (**Table 3.1**) numbering; GenBank accession: X99298). Multiple sequence alignment revealed partial 16S rDNA sequence similarity of 95.4-100% among all 22 strains and revealed partial 16S rDNA sequence similarity of 98.0-100 % among the three putative *X. t. g* isolates, the three putative *X. t. poae* isolates and the 15 reference *Xanthomonas* strains (without *Stenotrophomonas maltophilia*). Despite the high degree of 16S rDNA sequence similarity among the three putative *X. t. g* isolates, the three putative *X. t. poae* isolates and the 15 reference *Xanthomonas* strains, a 50-bp signature sequence (**Figure 3.1**) was identified, which distinguishes the putative *X. t. g* isolates, r3, and the reference *Xanthomonas* strains other than *X. translucens* from group V (**Figure 3.1**) which consists of the putative *X. t. g* isolates, r0 and r5, the three putative *X. t. poae* isolates and all the reference *X. translucens* strains. The signature sequence also divides the putative *X. t. g* isolate, r3, and the reference *Xanthomonas* strains other than *X. translucens* into 4 groups (I, II, III, IV) (**Figure 3.1**). The signature sequence of each group was designated as SS (signature sequence) I, SS II, SS III, SS IV and SS V, respectively.

Phylogenetic analysis identified two main clades with bootstrap values of 68% and 97%, respectively (clade I and clade II, respectively; **Figure 3.2**).

Clade I **Figure 3.2** consists of the putative *X. t. g* isolate, r3, *X. oryzae pv. oryzae(T)*, *X. campestris pv. campestris(T)*, *X. bromi(T)* and *X. cassavae(T)* (*r3*, *X. o. o (T)*, *X. c. c (T)*, *X. bromi (T)* and *X. cassavae (T)*, respectively). These five strains also share the same signature sequence,

SS I **Figure 3.1**.

Clade II **Figure 3.2** is consisted of the putative *X. t. g* isolates, r0 and r5, the three putative *X. t. poae* isolates and all the reference *X. translucens* strains. These thirteen strains also share the same signature sequence, SS V **Figure 3.1**. *X. hyacinthi(T)* carrying SS II and *X. melonis(T)* carrying SS II are clustered together in a clade **Figure 3.2** that is separated from clade I and clade II. Both *X. albilineans(T)* carrying SS III **Figure 3.1**, **Figure 3.2** and *X. sacchari(T)* carrying SS IV **Figure 3.1**, **Figure 3.2** are separated from clade II and the clade that consists of *X. hyacinthi(T)* and *X. melonis(T)* but both *X. albilineans(T)* and *X. sacchari(T)* are nested with the two clades in clade III. *S. maltophilia(T)* is the outgroup.

In clade II, the putative *X. t. g* isolates, r0 and r5, and all the reference *X. t. g* strains are clustered in subclade II(*g*) **Figure 3.2** while the three putative *X. t. poae* isolates, the reference *X. t. poae* strains, the reference *X. t. t (T)* and the reference *X. t. undu(T)* are clustered in subclade II(*poae*) **Figure 3.2**. There is no heterogeneity among the partial 16S rDNA sequences of the putative *X. t. g* strains, r0 and r5, and the reference *X. t. g* strains (subclade II(*g*), **Figure 3.2**). The sequence similarity among the partial 16S rDNA sequences of these strains cluster in subclade II(*poae*) is 99.9-100% due to the nucleotide variances at a single locus (position 1242, *X. t. g(T)* **Table 3.1** numbering; GenBank accession: X99298). The nucleotide at this locus is C (cytosine) in the partial 16S rDNA sequence of *X. t. t(T)*, *X. t. undu(T)* and the three putative *X. t. poae* isolates. The nucleotide at this locus is missing in the partial 16S rDNA sequence of *X. t. poae (T)* while it is A (adenine) in the partial 16S rDNA sequence of *X. t. poae (1)*.

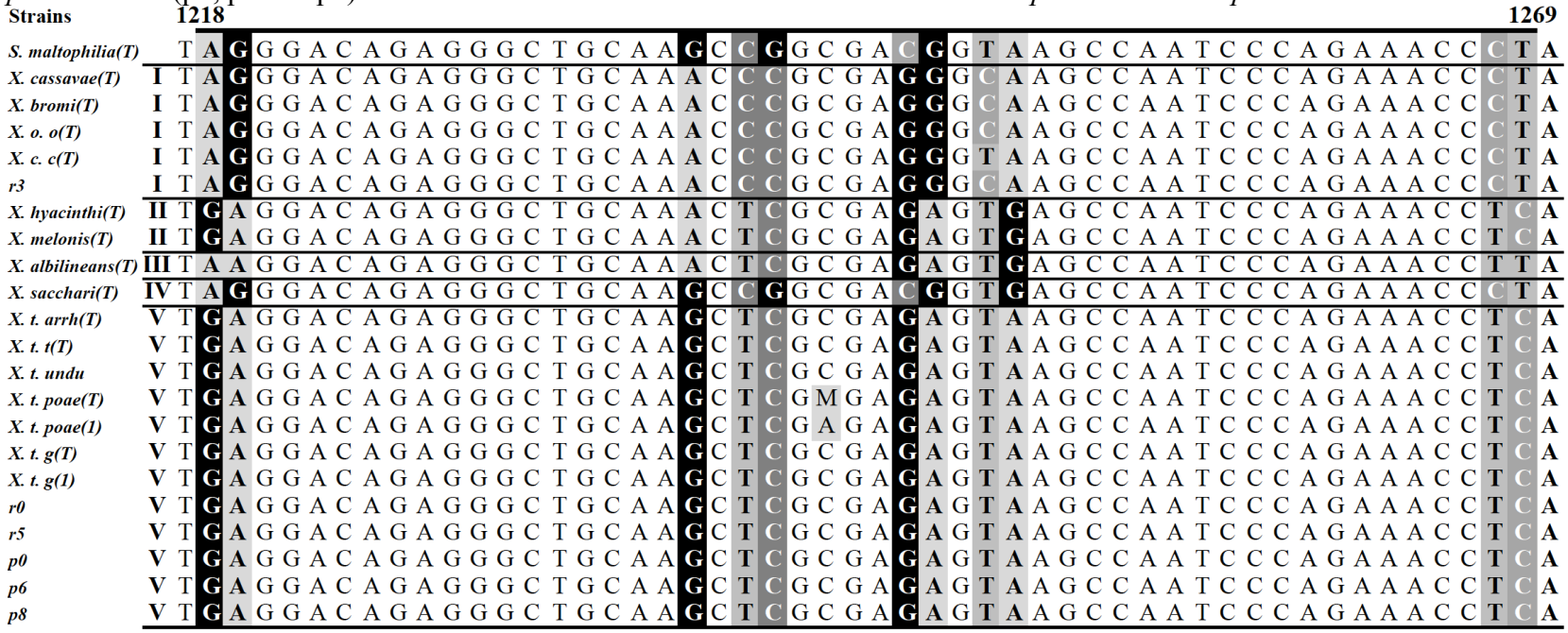
Three signature loci (position: 251, 1111 and 1242, *X. t. g(T)* **Table 3.1** numbering; GenBank accession: X99298) are identified and they separate subclade II(*g*) **Figure 3.2** from subclade II(*poae*) **Figure 3.2**. For all the strains (r0, r5, *X. t. g (T)* and *X. t. g (1)*) that cluster in subclade

II(*g*), the three nucleotides at the three loci are A, A and C, respectively. For the strains (p0, p6, p8, *X. t. t (T)* and *X. t. undu*) that cluster in subclade II(*poae*) but not including *X. t. poae(T)* and *X. t. poae (1)*, the three nucleotides at the three loci are G (guanine), C and C, respectively. For *X. t. poae(T)*, the three nucleotides at the three loci are G, C and M (M: nucleotide missing), respectively. For *X. t. poae (1)*, the three nucleotides at the three loci are G, C, A, respectively.

Two signature loci (position: 251 and 318, *X. t. g(T)* **Table 3.1** numbering; GenBank accession: X99298) separate *X. t. arrh(T)* from subclade II(*g*). The two nucleotides at the two loci are G and G, respectively, in the partial 16S rDNA sequence of X. *t. arrh(T)* while are A and A, respectively, in the partial 16S rDNA sequence of strains (r0, r5, *X. t. g (T)* and *X. t. g (1)*) in subclade II(*g*).

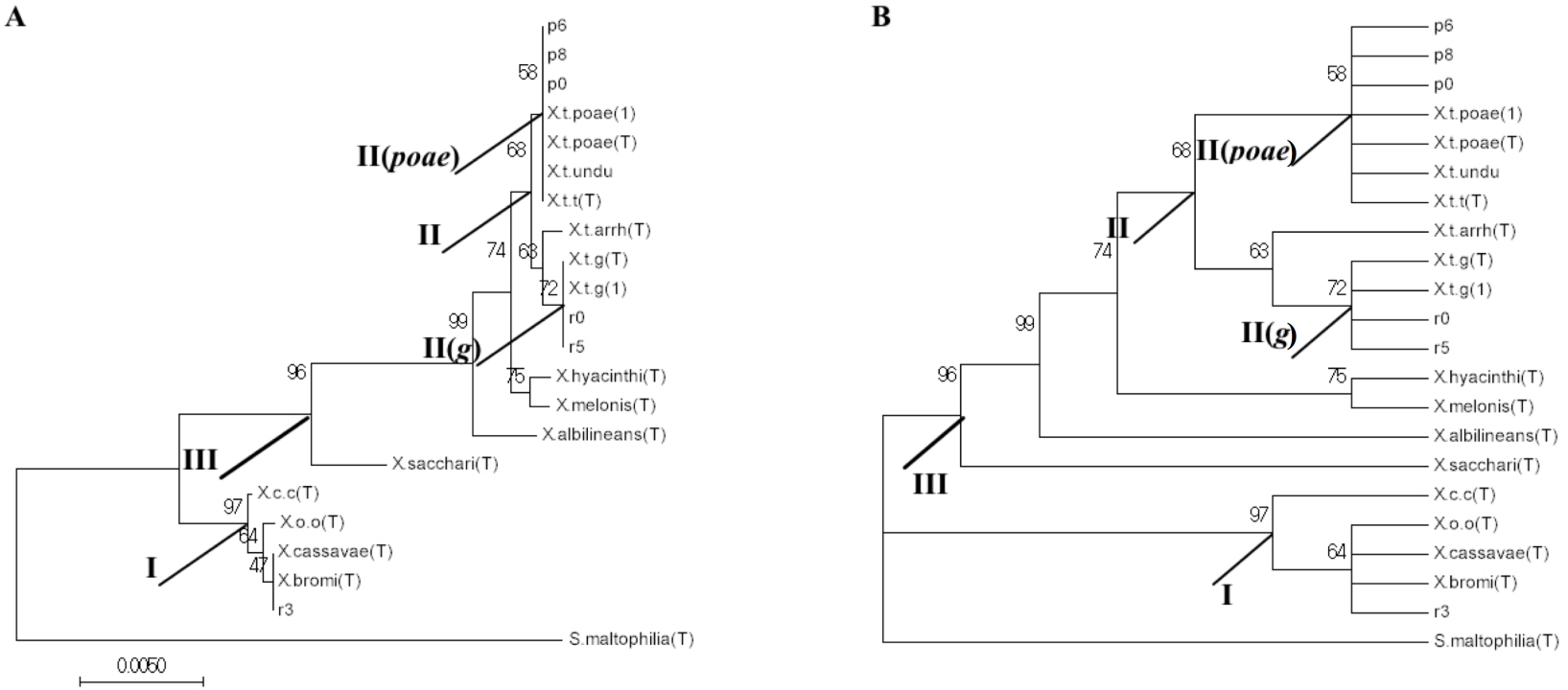
Consequently, the analysis of partial 16s rDNA sequences confirmed the bacterial isolates, r0 and r5 are *X. t. g*, and confirmed the bacterial isolates, p0, p6 and p8, are *X. translucens*, while it failed to confirm the identity of bacterial isolate, r3, at species level.

Figure 3.1. Multi-alignment of partial 16S rDNA sequences of the three putative *X. t. g* isolates (r0, r3 and r5), the three putative *X. t. poae* isolates (p0, p6 and p8) and 15 the reference *Xanthomonas* strains and *Stenotrophomonas maltophilia*.



The bar on the top marks the 50-bp signature sequence. Numbers on the top denote the position in the *X. translucens pv. graminis* type strain LMG728 (*X. t. g(T)*) sequence. The Roman numerals, I, II, III, IV, V, indicate the five groups referred in the text.

Figure 3.2 (A, B). **A**. The phylogram of the neighbor-joining tree based on the multi-alignment of the partial 16S rDNA sequences of the three putative *X. t. g* isolates, the three putative *X. t. poae* isolates, the reference strain, *Stenotrophomonas maltophilia*, and the 15 reference *Xanthomonas* strains. **B**. The cladogram of the neighbor-joining tree based on the multi-alignment of the partial 16S rDNA sequences of the three putative *X. t. g* isolates, the three putative *X. t. poae* isolates, the reference strain, *Stenotrophomonas maltophilia*, and the 15 reference *Xanthomonas* strains.



In **A**, the "(T)" indicates the type-strain. The "(1)" indicates the strain other than the type strain. Numbers on nodes indicate bootstrap values (%) that were obtained from 1000 replicates. I, II, I(*g*) and I(*poae*) indicate the major clades referred to the text. In **B**, the "(T)" indicates the type-strain. The "(1)" indicates the strain other than the type strain. Numbers on nodes indicate bootstrap values (%) that were obtained from 1000 replicates. Nodes with bootstrap values (%) lower than 50% were collapsed to polytomies. I, II, I(*g*) and I(*poae*) indicate major clades referred to in the text.

Analysis of multilocus sequences

The four housekeeping genes, *gyrB*, *fusA*, *gapA* and *lepA*, were successfully amplified from the six bacterial isolates, r0, r3, r5, p0, p6 and p8, respectively. Sequences of each of the housekeeping genes were multi-aligned with MUSCLE [13] embedded in MEGA7 [14] using the default parameters and both ends of each alignment were trimmed to the following sizes: *gyrB* (411 bp), *fusA* (558 bp), *gapA* (444 bp) and *lepA* (390 bp). The trimmed sequences were concatenated to give a total length of 1803 bp.

The analysis of multilocus sequences identified two main clades with bootstrap values of 98% and 100% (clade 1 and clade 2; **Figure 3.3**).

Clade 1 consists of all the bacterial species (r0, r5, p0, p6, p8, *X. translucens pv. graminis*, *X. translucens pv. poae*, *X. translucens pv. undulosa*, *X. translucens pv. arrhenatheri* and *X. translucens pv. translucens*) that cluster in clade III in the analysis of partial 16S rDNA sequences (**Figure 3.2**, clade III) except for *X. melonis* (sequences of housekeeping genes of *X. melonis* couldn't be found in databases). In clade 1, the *X. t. g* isolates, r0 and r5, the three putative *X. t. poae* isolates and all reference *X. translucens* strains cluster together **Figure 3.3** while they are separated from *X. albilineans*, *X. hyacinthi* and *X. sacchari*, which is consistent with the analysis of partial 16S rDNA sequences. In clade 1, the *X. t. g* isolates, r0 and r5, and all the reference *X. t. g* strains cluster in subclade 1(*g*) **Figure 3.3b**, which is consistent with the analysis of partial 16S rDNA sequences. However, the evolutionary relationship among *X. t. poae (1)*, *X. t arrh (T)* and subclade 1(*g*) (r0, r5, *X. t. g (T)* and *X. t. g (1)*) is obscure **Figure 3.3b**, which is not congruent with the analysis of partial 16S rDNA sequences. The analysis of partial 16S rDNA sequences revealed subclade II(*g*) (r0, r5, *X. t. g (T)* and *X. t. g (1)*) and *X. t arrh (T)* have a closer relationship **Figure 3.2B** while subclade II(*g*) (r0, r5, *X. t. g (T)* and *X. t. g (1)*) and *X. t. poae (1)* have a further

relationship **Figure 3.2B**.

Consequently, the analysis of multilocus sequences confirmed again that bacterial isolates, r0 and r5, are *X. t. g*.

The polytomy, subclade II(*poae*), **Figure 3.2B** in the analysis of partial 16S rDNA sequences is partially solved in the analysis of multilocus sequences. The analysis of multilocus sequences revealed that *X. t. poae(T)* and the three putative *X. t. poae* isolates are clustered in subclade 1(*poae*) **Figure 3.3b**. In subclade 1(*poae*), it clearly revealed that *X. t. poae(T)* and p0 have the closest evolutionary relationship **Figure 3.3b**, *X. t. poae(T)* and p6 have a further evolutionary relationship **Figure 3.3b** and *X. t. poae(T)* and p8 have the furthest evolutionary relationship **Figure 3.3b**. Analysis of multilocus sequences also separated *X. t. undu(T)* and *X. t. t(T)* from subclade 1(*poae*) **Figure 3.3b**. But, the evolutionary relationship between subclade 1(*poae*) and *X. t. poae* (1) is still obscure.

Consequently, analysis of multilocus sequences confirmed that the three putative *X. t. poae* isolates are *X. t. poae* and they have a closer evolutionary relationship to *X. t. poae(T)*.

The analysis of multilocus sequences revealed that *S. maltophilia(T)* is still the outgroup of clade 1 (r0, r5, p0, p6, p8, *X. translucens pv. graminis*, *X. translucens pv. poae*, *X. translucens pv. translucens*, *X. translucens pv. undulosa*, *X. translucens pv. arrhenatheri X. albilineans, X. sacchari* and *X. hyacinthi*) **Figure 3.3**. It is consistent with the analysis of partial 16S rDNA sequences that *S. maltophilia(T)* is the outgroup of clade III (r0, r5, p0, p6, p8, *X. translucens pv. graminis*, *X. translucens pv. poae*, *X. translucens pv. translucens*, *X. translucens pv. undulosa*, *X. translucens pv. arrhenatheri X. albilineans, X. sacchari, X. melonis* and *X. hyacinthi*) **Figure 3.2**. However, *S. maltophilia* is not the outgroup of genus *Xanthomonas* any more in the analysis of multilocus sequences.

The analysis of multilocus sequences revealed 71.8-97.2% sequence similarity **Table 3.5** among inter-species groups and revealed 97.5-100% internal species sequence similarity.

The bacterial isolate, r3, *X. bromi(T)*, *X. ory. ory(T)*, *X. c. c(T)*, and *X. cassavae(T)* cluster in clade 2 **Figure 3.3**. The analysis of multilocus sequences still failed to assign bacterial isolate, r3, to any species group in clade 2 **Figure 3.3**. Bacterial isolate, r3, has 93.8-95.6% sequence similarity to other species groups in clade 2 **Figure 3.3** and has the highest sequence similarity, 95.6%, to *X. vas*** (*X. vasicola*) **Figure 3.3**, **Table 3.5**.

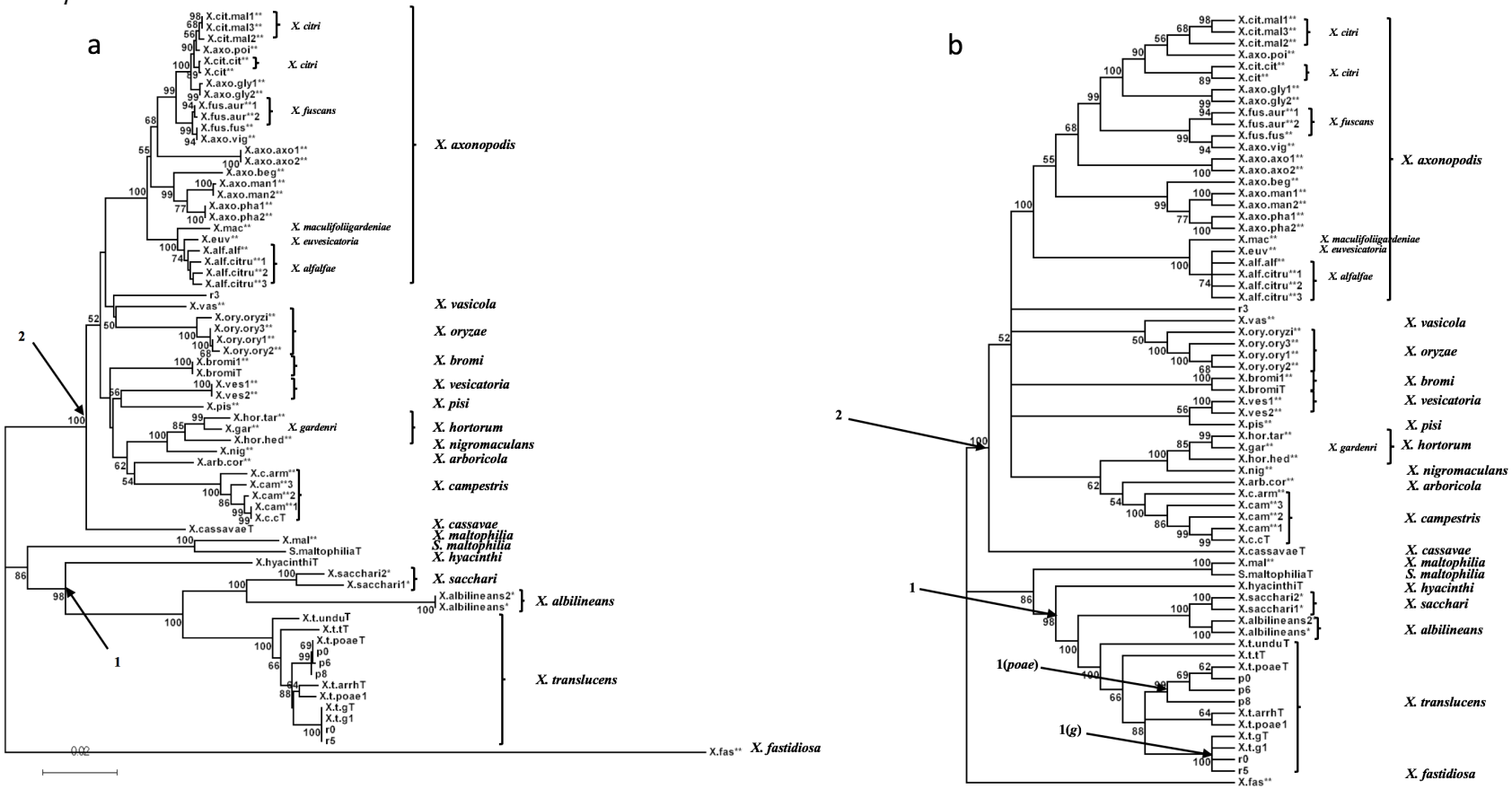
Multi-alignment revealed that the sequence similarity is 100% among the strains in subclade 1(*g*) and is 99.9%-100% among the strains in subclade 1(*poae*). The heterogeneities among multilocus sequences of strains in subclade 1(*poae*) are due to the nucleotide variances at three different loci **Figure 3.4 a**. Multilocus sequence similarity among *X. t. arrh (T)* and the strains in subclade 1(*g*) is 98.3-100% due to nucleotide variances at 23 difference loci **Figure 3.4 b**. Multilocus sequence similarity among *X. t. poae (1)* and the strains in subclade 1(*poae*) was 98.9-99% due to nucleotide differences at 21 different loci **Figure 3.4 c**. Multilocus sequence similarity among *X. t. g* isolates, r0 and r5, and the three putative *X. t. poae* isolates was 98.6-100% due to nucleotide difference at 27 different loci **Figure 3.4 d**.

Table 3.5. Distance matrix among inter-species groups constructed based on analysis of multilocus sequences.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *X.vasicola*																		
2. *X.translucens*	0.134																	
3. *X.sacchari*	0.142	0.075																
4. *X.pisi*	0.054	0.137	0.138															
5. *X.vesicatoria*	0.045	0.136	0.142	0.058														
6. *X.nigromaculans*	0.055	0.134	0.143	0.053	0.058													
7. *X.maltophilia*	0.124	0.153	0.126	0.122	0.131	0.128												
8. *X.hyacinthi*	0.122	0.1	0.139	0.118	0.121	0.116	0.134											
9. *X.hortorum*	0.056	0.137	0.147	0.054	0.061	0.028	0.128	0.118										
10. *X.fastidiosa*	0.242	0.28	0.282	0.238	0.243	0.241	0.262	0.261	0.239									
11. *X.cassavae*	0.059	0.135	0.138	0.06	0.062	0.062	0.119	0.117	0.065	0.238								
12. *X.campestris*	0.064	0.138	0.144	0.063	0.07	0.053	0.123	0.119	0.059	0.24	0.059							
13. *X.bromi*	0.052	0.137	0.143	0.047	0.058	0.053	0.126	0.12	0.055	0.248	0.054	0.056						
14. *X.axonopodis*	0.048	0.138	0.141	0.056	0.056	0.06	0.128	0.121	0.063	0.242	0.058	0.07	0.053					
15. *X.arboricola*	0.05	0.13	0.134	0.048	0.05	0.044	0.124	0.107	0.048	0.244	0.05	0.047	0.049	0.054				
16. *X.albilineans*	0.174	0.104	0.074	0.168	0.17	0.171	0.159	0.163	0.169	0.272	0.166	0.176	0.174	0.169	0.167			
17. *S.maltophilia*	0.127	0.158	0.127	0.128	0.133	0.127	0.047	0.137	0.132	0.259	0.117	0.129	0.126	0.129	0.123	0.156		
18. *r3*	0.044	0.135	0.144	0.053	0.052	0.06	0.124	0.118	0.062	0.241	0.054	0.062	0.053	0.054	0.047	0.173	0.13	

Xanthomonas citri, *Xanthomonas fuscans*, *Xanthomonas maculifoliigardeniae*, *Xanthomonas euvesicatoria*, *Xanthomonas alfalfae* are included in *X. axonopodis*, so, they are not listed in this table. r0, r5, p0, p6 and p8 are included in *Xanthomonas translucens*, so they are not listed in this table.

Figure 3.3 (a, b). **a**. Phylogram of the neighbor-joining tree based on the multi-alignment of concatenated multilocus sequences (*gyrB+fusA+gapA+lepA*) of r3, the *X. t. g* isolates, r0 and r5, the three putative *X. t. poae* isolates, *Stenotrophomonas maltophilia* and 60 reference *Xanthomonas* strains. **b**. Cladogram of the neighbor-joining tree based on the multi-alignment of concatenated multilocus sequences (*gyrB+fusA+gapA+lepA*) of r3, the *X. t. g* isolates, r0 and r5, the three putative *X. t. poae* isolates, *Stenotrophomonas maltophilia* and 60 reference *Xanthomonas* strains.



In **a**, numbers on nodes indicate bootstrap values (%) that were obtained from 1000 replicates. "1" and "2" indicate the major clades referred to the text. Species names shown on the right side of the trees represent the species groups. In **b**, numbers on nodes indicate bootstrap values (%) that were obtained from 1000 replicates. Nodes with bootstrap value (%) lower than 50% were collapsed to polytomies. "1", "2", "1(*g*)" and "1(*poae*)" indicate the major clades referred to the text. Species names shown on the right side of the trees represent the species groups.

Figure 3.4. Nucleotide variances in concatenated sequence of housekeeping genes.

a

Strains	428	455	701
X.t.poae(T)	-	T	G
p0	-	T	G
p6	A	T	A
p8	T	C	G

b

Strains	63	90	202	214	261	348	552	555	689	848	962	968	1076	1093	1112	1211	1283	1289	1352	1391	1502	1508	1809
X.t.arrh(T)	C	C	C	T	G	C	C	G	C	C	C	T	C	A	C	T	G	A	C	G	T	C	T
r0	A	T	T	C	C	G	T	A	T	A	T	C	T	G	G	C	C	C	G	C	C	T	C
r5	A	T	T	C	C	G	T	A	T	A	T	C	T	G	G	C	C	C	G	C	C	T	C

c

Strains	288	312	336	348	428	455	701	1077	1121	1166	1211	1283	1346	1391	1454	1508	1547	1625	1700	1709	1860
X.t.poae(1)	C	C	G	G	-	T	G	C	A	T	T	G	C	G	G	C	C	G	C	G	T
p0	T	T	A	C	-	T	G	A	G	C	C	C	T	C	C	T	G	C	G	C	C
p6	T	T	A	C	A	T	A	A	G	C	C	C	T	C	C	T	G	C	G	C	C
p8	T	T	A	C	T	C	G	A	G	C	C	C	T	C	C	T	G	C	G	C	C

d

Strains	63	90	202	288	312	336	348	428	455	552	555	579	689	701	780	848	962	1076	1093	1112	1162	1166	1199	1346	1352	1625	1700
r0	A	T	T	C	C	G	G	-	T	T	A	T	T	G	A	A	T	T	G	G	G	T	T	C	G	G	C
r5	A	T	T	C	C	G	G	-	T	T	A	T	T	G	A	A	T	T	G	G	G	T	T	C	G	G	C
p0	C	C	C	T	T	A	C	-	T	C	G	C	C	G	G	C	C	C	A	C	C	C	C	T	C	C	G
p6	C	C	C	T	T	A	C	A	T	C	G	C	C	A	G	C	C	C	A	C	C	C	C	T	C	C	G
p8	C	C	C	T	T	A	C	T	C	C	G	C	C	G	G	C	C	C	A	C	C	C	C	T	C	C	G

a: Loci with nucleotide variances among *X. t. poae (T)* and strains in clade 1(*poae*). **b:** Loci with nucleotide variances among *X. t. arrh(T)* and strains in clade 1(*g*). **c:** Loci with nucleotide variances among *X. t. poae(T)* and strains in clade 1(*poae*). **d:** Loci with nucleotide variances among strains in clade 1(*g*) and strains in clade 1(*poae*). Numbers on the top denote position in the multi-aligned sequences.

DISCUSSION

Analysis of partial 16S ribosomal DNA sequences confirmed putative *X. t. g* isolates, r0 and r5, are *X. t. g*, the three putative *X. t. poae* isolates are *X. translucens*, and revealed that all the five bacterial isolates (r0, r5, p0, p6 and p8) and all reference *X. translucens* strains not only share the same signature sequence, SS V **Figure 3.1**, but also cluster together (clade II) **Figure 3.2**. The result is consistent with that of Mikowski et al. [11]. The high sequence similarity observed in clade II **Figure 3.2** is also consistent with high similarity generally observed among *X. translucens* pathovars based on membrane proteins [15], fatty acid profiling [16], ribosomal intergenic sequences [17] and 16S rDNA analysis [18].

Though analysis of partial 16S rDNA sequences failed to confirm the identity of the three putative *X. t. poae* isolates at infraspecific level, it grouped the three putative *X. t. poae* isolates with the reference *X. translucens pv. poae* strains (clade II(*poae*) **Figure 3.2**. However, the obscure evolutionary relationship among *X. translucens pv. undulosa*, *X. translucens pv. poae* and *X. translucens pv. translucens* (clade II(*poae*) **Figure 3.2** is not as specific as that in Mikowski et al. [11]. Mikowski et al. [11] conducted an analysis of 16-23S rDNA sequences (partial 16S, complete intergenic spacer region and partial 23S ribosomal DNA sequence) which not only grouped among *X. translucens pv. undulosa*, *X. translucens pv. poae* and *X. translucens pv. translucens* together but also clearly divided them into different subclades. The result of Mikowski et al. [11] suggests more genetic variances are exited in the intergenic spacer region and 23S rDNA among *X. translucens pv. undulosa*, *X. translucens pv. poae* and *X. translucens pv. translucens.*

Analysis of partial 16S ribosomal DNA sequences in this study confirmed that r3 is not *X. translucens* but failed to confirm its identity at species level **Figure 3.2**. The result that r3 is not *X. translucens* is consistent with previous study that r3 caused the least disease severity on both *L.*

perenne and *P. annua* when compared to the other five bacterial isolates (r0, r5, p0, p6 and p8).

The 50-bp signature sequence **Figure 3.1** separates strains (r0, r5, p0, p6, p8 and those reference *X. translucens* strains) in group V **Figure 3.1** from the strains (those reference *Xanthomonas* strains other than *X. translucens*) in group I-IV **Figure 3.1**. The result is congruent with the 25-bp signature sequence did in Kölliker et al. [7].

Three signature loci (positions: 251, 1111 and 1242, *X. t. g(T)* **Table 3.1** numbering; GenBank accession: X99298) at partial 16S rDNA sequence clearly separated subclade II(*g*) from subclade II(*poae*) **Figure 3.2**. However, nucleotide variances at position 1242 only restricted in *X. t. poae(T)* [7] and *X. t. poae(1)* (*X. t. poae* ATCC 33804, GenBank accession AY572961) **Table 3.1**. The nucleotide is missing at position 1242 in the partial 16S rDNA sequence of *X. t. poae(T)* and the nucleotide is A at position 1242 in the partial 16S rDNA sequence of *X. t. poae(1)*. Another partial 16S rDNA sequence of *X. t. poae* ATCC 33804 (GenBank accession: JN869509.1) [19] was multi-aligned with the partial 16S rDNA sequences of the strains in subclade II(*g*) and the strains in subclade II(*poae*). The result revealed that the nucleotide variances only exist at positions 251 and 1111 because the new partial 16S rDNA sequence of *X. t. poae* ATCC 33804 (GenBank accession: JN869509.1) is shorter and it doesn't contain position 1242. However, the new partial 16S rDNA sequences of *X. t. poae* ATCC 33804 (GenBank accession: JN869509.1) [19] and *X. t. poae(T)* [7] were amplified by the same primers [7, 19]. So, it is reasonable to hypothesis that the nucleotide variances at position 1242 in partial 16S rDNA sequence of *X. t. poae(T)* and *X. t. poae(1)* are due to experiment difficulty for sequencing gene at that length. So, the two signature loci (positions: 251 and 1111, *X. t. g(T)* **Table 3.1** numbering; GenBank accession: X99298) at partial 16S rDNA sequence can be used to rapidly distinguish *X. translucens pv. graminis* from *X. translucens pv. translucens*, *X. translucens pv. undulosa* and *X. translucens pv. poae*.

Consequently, 16S rDNA identification provides a rapid and reliable method for the identification of X. translucens pv. graminis but is not specific for the identification of *X. translucens pv. poae*. The two signature loci (position: 251 and 1111, *X. t. g(T)* **Table 3.1** numbering; GenBank accession: X99298) at partial 16S rDNA sequence can rapidly separate *X. translucens pv. graminis* from *X. translucens pv. translucens*, *X. translucens pv. undulosa* and *X. translucens pv. poae*.

The structure of genus *Xanthomonas* produced by neighbor-joining tree **Figure 3.3** based on concatenated sequences of four housekeeping genes (*gyrB*, *fusA*, *gapA* and *lepA*) in this study is largely consistent with the structure of genus *Xanthomonas* produced by neighbor-joining tree based on partial 16S rDNA sequences in this study, in Kölliker et al. [7], and in Hauben et al. [20], and is also largely consistent with the structure of genus *Xanthomonas* produced by neighbor-joining tree based on concatenation of housekeeping genes (*dnaK*, *fyuA*, *gyrB* and *rpoD*) in Young et al. [21]. All studies revealed that genus *Xanthomonas* consists of two distinct groups: (1) *X. albilineans*, *X. hyacinthi*, *X. sacchari* and *X. translucens* (**Figure 3.3**, clade 1) and (2) *X. arboricola*, *X. axonopodis*, *X. bromi*, *X. campestris*, *X. cassavae*, *X. hortorum*, *X. oryzae*, *X. pisi*, *X. vasicola* and *X. vesicatoria* (**Figure 3.3** clade 2).

The analysis of multilocus sequences in this study revealed that the *X. translucens* strains (including r0, r5, p0, p6 and p8) have a closer evolutionary relationship to *X. albilineans* (**Figure 3.3** clade 1) and have a further evolutionary relationship to *X. hyacinthi* (**Figure 3.3** clade 1) while the analysis of partial 16S rDNA sequences in this study, Kölliker et al. [7], Hauben et al. [20] and Young et al. [21] have the opposite result. It suggests that choices of genes can influence the structure of the tree.

Several polytomies exist in clade 2 **Figure 3.3 b** which indicates the analysis of multilocus

sequences in this study is not as specific as that in Young et al. [21] and that in Almeida et al. [22] for describing the evolutionary relationship among strains in clade 2 **Figure 3.3 b**. The existence of those polytomies may be due to reason that the four housekeeping genes used in this study are more conserved than those four housekeeping genes used in Young et al. [21]. It also suggests that more data (genes) are required for specifically describing the evolutionary relationship of the strains in clade 2 **Figure 3.3 b** because Almeida et al. [22] used not only the four housekeeping genes that were used in this study, but also used another two housekeeping genes, *gltA* and *lacF*, and more specifically described the evolutionary relationship among the strains in clade 2 **Figure 3.3 b**. Depending on the results of this study and Almeida et al. [22], it indicates that more genetic variabilities exist in housekeeping genes, *gltA* and *lacF,* among the strains in clade 2 **Figure 3.3 b**.

Though the four housekeeping genes don't clearly describe the evolutionary relationship among the strains in clade 2 **Figure 3.3**, they clearly describe the evolutionary relationship among strains in clade 1 **Figure 3.3**. The analysis of multilocus sequences in this study clearly separates *X. translucens pv. poae* from *X. translucens pv. translucens* and *X. translucens pv. undulosa* while the analysis of partial 16S rDNA in this study, in Kölliker et al. [7], and in Hauben et al. [17] failed to separate them, which indicates the concatenated sequence of the four housekeeping genes is more specific than 16S rDNA for describing the evolutionary relationship among *X. translucens pv. translucens* and *X. translucens pv. undulosa*.

The analysis of multilocus sequences also illustrated that *X. translucens pv. graminis* and *X. translucens pv. poae* have a closer evolutionary relationship while both *X. translucens pv. graminis* and *X. translucens pv. poae* have a further evolutionary relationship to *X. translucens pv. translucens* and *X. translucens pv. undulosa*. The result was not illustrated in Young et al. [21] and

in Almeida et al. [22].

Analysis of multilocus sequence revealed 71.8%-97.2% sequence similarity among inter-species groups and 97.5-100% internal species sequence similarity. In clade 2 **Figure 3.3**, r3 has the low inter-species sequence similarity, 93.8-95.6%, and it is not assigned to any species groups in clade 2, which suggests that r3 may be a new *Xanthomonas* species.

Multi-alignment analysis of concatenated sequences revealed 100% sequence similarity among *X. translucens pv. graminis* strains in subclade 1(*g*) **Figure 3.3**while it revealed sequence heterogeneity among p0, p6, p8, *X. translucens pv. poae (T)* and *X. translucens pv. poae (1)* in subclade *1(poae)* **Figure 3.3** which indicates the four housekeeping genes, *gyrB*, *fusA*, *gapA* and *lepA*, are extremely conserved in *X. translucens pv. graminis* while are more variable *in X. translucens pv. poae*.

CONCLUSION

The study demonstrated that r0 and r5 are *X. translucens pv. graminis,* p0, p6 and p8 are *X. translucens pv. poae* while r3 may be a new *Xanthomonas* species. The analysis of partial 16S rDNA in this study is specific enough to confirm the identity of *X. translucens pv. graminis* while is not specific enough to confirm the identity of *X. translucens pv. poae*. The multilocus analysis of concatenated sequences of four housekeeping genes in this study is specific to confirm the identity of *X. translucens pv. graminis* and *X. translucens pv. poae*. The two analyses in this study revealed that the genes used in this study are extremely conserved in *X. translucens pv. graminis* while are more variable in *X. translucens pv. poae* strains. Consequently, 16S rDNA can be used as a reliable genetic marker for rapid identification of *X. translucens pv. graminis* while the concatenated sequence of four housekeeping genes, *gyrB*, *fusA*, *gapA* and *lepA*, can be used as a reliable genetic marker for rapid identification of both *X. translucens pv. graminis* and *X.*

translucens pv. poae.

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