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GENETIC DISTANCE MAY UNDERLIE VIRULENCE DIFFERENCES AMONG ISOLATES OF A BACTERIAL PLANT PATHOGEN

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SUMMARY

Certain pathogens evolve with their hosts in a stepwise arms race of virulence and resistance, mediated by one or a few genetic loci in their genomes. Other pathogens may retain the ability to colonize hosts through multiple small genetic changes, which do not necessarily occur solely at loci under selective pressure. The bacterial plant pathogen *Xylella fastidiosa* lacks classic indicators of pathogenicity such as a type III secretion system, which makes the identification of its virulence mechanism challenging. We tested the hypothesis that quantitative differences in virulence between pairs of *X. fastidiosa* isolates were correlated with their respective genetic distances. Isolates from two different *X. fastidiosa* subspecies were genetically typed using 20 loci, generating information on the genetic distance among pairs of isolates. Virulence data were obtained by determining the impact of these genotypes on the phenotype of alfalfa plant hosts. Results show significant or marginally significant correlations with respect to differences in infection level and alfalfa stunting, suggesting that genetic distance partially explains plant phenotypic differences in virulence among *X. fastidiosa* isolates. Furthermore, the data demonstrate substantial amount of phenotypic variation among *X. fastidiosa* isolates within subspecies *fastidiosa* and *multiplex*.

Key words: Xyelella fastidiosa, Pierce's disease, sharpshooter vector, xylem-limited, alfalfa

INTRODUCTION

Genotypic characterization of bacterial plant pathogens has revealed that genetic clusters share phenotypic characteristics (e.g. Morris *et al*., 2010). One implication is that distinct phylogenetic clades may differentially cause disease on specific hosts. In plant pathology, specifically, bacterial pathogenicity to host plants is often linked to a single-gene arms race, in which plant resistance genes co-evolved with pathogen effector-encoding genes (Nimchuk *et al*., 2003). In this scenario, evolution of pathogenicity is tightly linked to one locus, or set of loci, rather than whole-genome evolutionary change (Lindeberg *et al*., 2009). As such, horizontal transfer of effectors can result in emergence of new pathogen lineages in otherwise non-pathogenic genetic backgrounds (Ma *et al*., 2006). Beyond effectors, other genetic differences exist among biologically similar isolates (e.g. within strains or pathovars), which could be linked in a quantitative fashion to differences in pathogenicity. In other words, isolates may be similar in genetic and biological characteristics, with minor phenotypic differences linked to genome-wide variability rather than to specific loci. This scenario is more likely to occur with pathogens that do not interact directly with live host cells and, for example, lack a type III secretion system and effectors, such as *X. fastidiosa* and other vascular bacteria. We tested this hypothesis with the economically important plant pathogen *X. fastidiosa*, which colonizes and causes disease in grapevines and almond trees, among other hosts. *X. fastidiosa* subspecies *fastidiosa* causes disease in grape and almond plants in the field, while subspecies *multiplex* causes disease in almond but not grape; these two subspecies are genetically distinct (Almeida *et al*., 2003). Unlike many other bacterial plant pathogens, *X. fastidiosa* exclusively colonizes plant xylem vessels, which are devoid of live cells. It is possible that *X. fastidiosa*-produced molecules reach live plant cells, but there is no evidence for this to date. Therefore, it is plausible that plant pathogenicity is not linked to a single locus, but instead is based on multiple genes associated with plant colonization. Disease symptoms of plants infected by *X. fastidiosa* are most likely a consequence of mechanical vessel clogging followed by progressive water stress, often resulting in characteristic leaf scorching (Chatterjee *et al*., 2008). In fact, it was recently demonstrated that an isolate of *X. fastidiosa* causes disease in a novel host upon modification of gene regulation (Killiny and Almeida, 2011). In this previous study, the difference between wild type and knockout mutant was quantitative, in that the same set of

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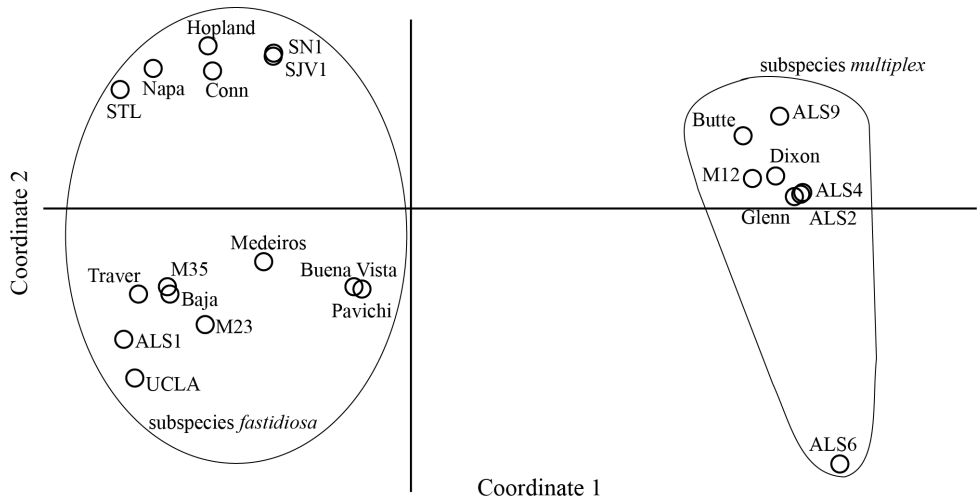

Fig. 1. Principal component analysis plot for all isolates tested in this study, indicating the genetic separation between *X. fastidiosa* subspecies *fastidiosa* and subspecies *multiplex*.

genes were present in both isolates, but regulated in a dissimilar fashion. Thus, *X. fastidiosa* is a suitable candidate to test the hypothesis that genetic distances among isolates sharing biological characteristics, such as host range, are reflected in phenotypic differences of infected hosts.

MATERIALS AND METHODS

***Xylella fastidiosa* strains and host phenotype.** The dataset was composed of 15 subspecies *fastidiosa* and 8 subspecies *multiplex* isolates previously investigated (Lopes *et al*., 2010). All isolates from subspecies *fastidiosa* were collected in California (1-ALS1, 2-Baja#5, 3-Buena Vista, 4-Conn, 5-Hopland, 6-M23, 7-M35, 8-Medeiros, 9-Napa, 10-Pavichi, 11-SJV1, 12-SN1, 13-STL, 14-Traver, 15-UCLA), except for Baja#5 (from Mexico). Isolates from subspecies *multiplex* were also collected in California (1-ALS2, 2-ALS4, 3-ALS6, 4-ALS9, 5-Butte, 6-Dixon, 7-Glenn, 8-M12). Seven phenotypic parameters (i.e. plant height, aboveground biomass, internode length, leaf blade width, leaf blade length, number of stems, and number of nodes) were previously characterized for healthy and *X. fastidiosa*-infected alfalfa plants (*Medicago sativa*) for each of the 23 isolates at four sampling periods, 8, 14, 21, and 35 weeks post inoculation (Lopes *et al*., 2010). *X. fastidiosa* infection in alfalfa results in plant stunting and other easily quantifiable disease phenotypes. In comparison, the degree of leaf scorching in grapevine and almond hosts is more difficult to quantify accurately.

Genotyping of *X. fastidiosa*. The 23 isolates previously used by Lopes *et al.* (2010) were genetically typed with 20 simple sequence repeat (SSR) markers described by Lin *et al.* (2005). These SSR loci were amplified in multiplexed reactions contained four sets of primers whereas the 6FAM (blue), NED (yellow), PET (red), and VIC (green) fluorescence dyes were used for labeling the forward primers. These markers allow for discrimination of *X. fastidiosa* isolates at the strain level (Coletta-Filho *et al*., 2011), which is not possible with DNA sequence-based markers such as multi-locus sequence typing (Almeida *et al*., 2008; Nunney *et al*., 2010). For the reactions, concentration and amplification methodologies followed exactly the conditions previously described (Coletta-Filho *et al*., 2011).

Data analyses. Genodive (Meirmans and Van Tienderen, 2004) was used to calculate the genetic differentiation G_{ST} index (Nei *et al*., 1987), number of effective genotypes (Eff_num), and genetic diversity (H_{Nei}) within each population. A principal coordinate analysis (PCoA) was performed to spatially visualize isolate genetic variation by using the GenAlEx v6.3 (Peakall and Smouse, 2006). CONVERT (Glaubitz, 2004) was used to estimate the allelic frequency among samples, used to obtain a Cavalli-Sforza chord distance matrix (with GENDIST in the PHYLIP package (Felsenstein, 1989). Phenotypic diversity among isolates was compared using a permutation multivariate analysis of variance (adonis() in the R programming language) with infection level [$\log_{10}$[(CFU/g of plant material)] and relative plant dry mass (g; as a % of healthy control plants) as response variables and pathogen isolate defined as a fixed effect. Given the expected differences among *X. fastidiosa* subspecies (Daugherty *et al*., 2010; Lopes *et al*., 2010), separate analyses were conducted for each subspecies. The Mantel test was used to evaluate the relationship between isolate pairwise genetic and phenotypic distances by providing a measure of correlation between two matrices (Sokal and Rohlf, 1995). Cavalli-Sforza chord distance values were related to each of the two metrics of plant virulence, separately: (i) differences in mean plant stunting (i.e. PC#1), and (ii) differences in mean infection level for pairs of isolates. Tests were conducted separately for grape and almond isolates,

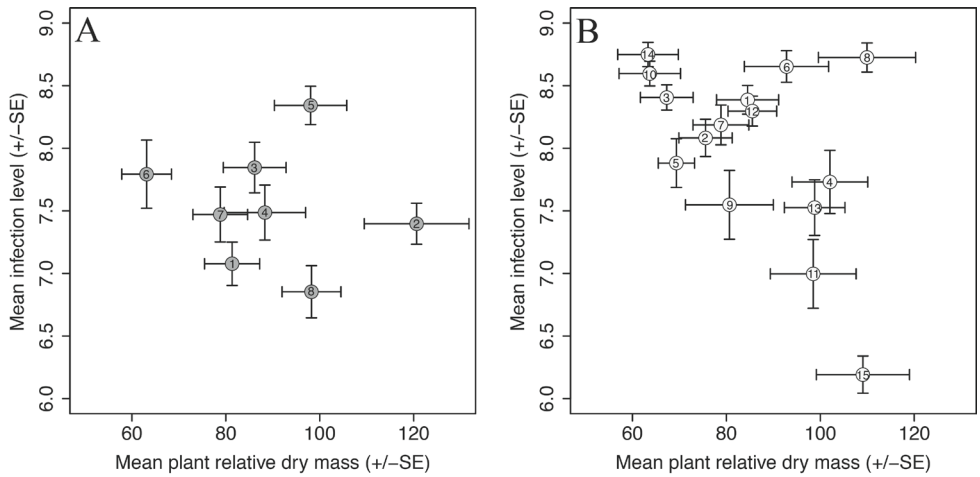

Fig. 2. Phenotypic diversity of alfalfa plants infected with A) one of 8 different *Xylella fastidiosa* subspecies *multiplex* or B) one of 15 *X. fastidiosa* subspecies *fastidiosa* isolates. Infection level noted as $\log_{10}$ (CFU/g of plant material). Plant mass is a relative measure (%) of the infected plant compared to healthy control plants. Numbers for individual isolates matches those listed in the Materials and Methods section.

with at least 999 permutations for each of the tests (function "mantel.rtest()" in the R programming language).

RESULTS AND DISCUSSION

Principal component analysis of simple sequence repeat (SSR)-based typing grouped the isolates into two groups, with all of those from subspecies *fastidiosa* and *multiplex* clearly clustered based on subspecies (Fig. 1) as previously reported with other molecular markers and analyses (Hendson *et al*., 2001; Almeida *et al*., 2003). A significant *Gst* value (0.26, $P < 0.001$), which is analogue to Wright's *Fst* index, reinforced the genetic isolation of these two clusters (i.e. subspecies). The number of effective genotypes within each group was the highest possible (*X. fastidiosa* subspecies *multiplex* = 8; *X. fastidiosa* subspecies *fastidiosa* = 15; no clones were observed), and consequently H_{Nei} genetic diversity values were also high (0.65 and 0.69, respectively), with no statistical difference between both. SSRs are fast-evolving markers based on tandem sequence repeats found across the *X. fastidiosa* genome (Coletta-Filho *et al*., 2001) and have been successfully used for genetic differentiation at the population level, which was our goal in this study, while SSRs were shown to not be adequate at higher levels such as subspecies (Almeida *et al*., 2008; Yuan *et al*., 2010). Using multi-locus sequence typing (sequence data from seven housekeeping loci), isolates within subspecies *fastidiosa* in the USA were shown to have very low levels of genetic diversity (nucleotide polymorphism estimated at 0.048%), compared to subspecies *multiplex* (0.240%; Yuan *et al*., 2010). Therefore, the degree of resolution obtained in this study was adequate to analyze within-subspecies genetic diversity. Accordingly, the remaining analyses considered the two subspecies separately.

The analyses of phenotypic diversity showed significant differences among isolates of both subspecies *fastidiosa* ($F_{14,367} = 3.893$, $P = 0.001$) and subspecies *multiplex* ($F_{7,159} = 3.552$, $P = 0.005$). The eight isolates of subspecies *multiplex* showed a range in both mean infection level and, especially, mean relative dry mass (Fig. 2A). The 15 isolates of subspecies *fastidiosa* showed a range in mean infection level from approximately 6 to nearly 9 $\log_{10}$ (CFU/g) and reductions in mass relative to controls between 0 and nearly 40% on average (Fig. 2B).

Because *X. fastidiosa* populations of each subspecies were genetically and phenotypically variable, correlations between genetic and phenotypic distances among pairs of isolates within each subspecies were performed. For subspecies *fastidiosa* there was a significant positive correlation for plant stunting ($r = 0.256$, $P = 0.042$) and a marginally significant positive correlation for infection level [$r = 0.222$, $P = 0.055$ (Fig. 3)]. Analyses of subspecies *multiplex* showed a positive but non-significant correlation between genetic distance and between-strain differences in plant stunting ($r = 0.387$, $P = 0.1$) and infection level ($r = 0.353$, $P = 0.117$). Although based on a relatively small sample size for one of the subspecies, these results broadly suggest that isolates more closely related genetically are also phenotypically more similar in virulence. We note that within subspecies *fastidiosa* in the USA, populations have very little genetic variation at the sequence level (Yuan *et al*., 2010). Therefore, we propose that evolutionary processes acting on populations with the degree of diversity analyzed here are rather recent in time.

The observed relationship between genotypic and phenotypic distance between pairs of isolates observed in this paper indicates that plant colonization and subsequent pathogenicity of *X. fastidiosa* subspecies may be a quantitative rather than qualitative trait. If true, a host-pathogen coevolutionary arms race would not be a strong selective pressure on *X. fastidiosa*, resulting in the decoupling of plant pathogenicity from an individual locus or a few loci. This hypothesis needs to be tested by comparing multiple

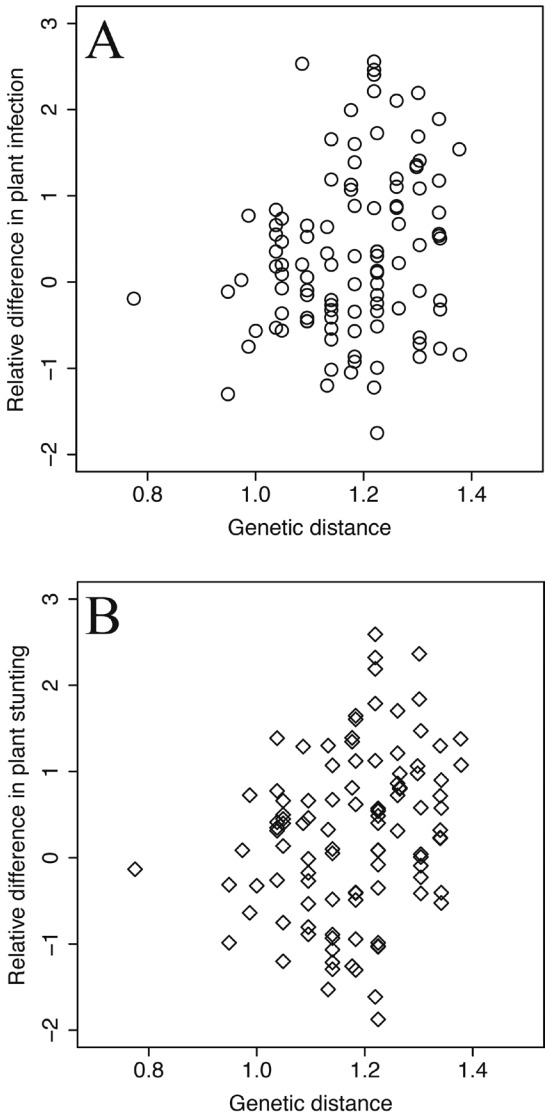

Fig. 3. Relative difference in alfalfa plant A) infection level (P = 0.055) and B) stunting (P = 0.042) induced by 15 isolates of the *Xylella fastidiosa* subspecies *fastidiosa* as a function of genetic distance between pairs of isolates. All 105 unique pairwise differences are shown. Infection is expressed as the difference in mean bacterial populations ($\log_{10}$ CFU/g of plant tissue) between each isolate. Stunting is expressed as the difference between mean values of the first principal component for 7 plant morphological measures, which includes primarily reductions in height, aboveground dry mass, and internode length.

X. fastidiosa genomes, in addition to large-scale phenotypic studies with more isolates. Results presented here, however, raise interesting questions about *X. fastidiosa* biology and evolution, some of which have applied consequences. If plant pathogenicity is a quantitative trait, phenotypic characterization of isolates linked with genome sequence comparisons may result in a more thorough understanding of how this bacterium colonizes plants and what factors are more or less important for successful colonization. In addition, management of resistant plant genotypes, obtained through traditional breeding or genetic engineering, should incorporate this aspect of *X. fastidiosa* biology and evolution into the development of control strategies, as loss of resistance may occur through different mechanisms than those of bacteria interacting with plants via effectors (Stall *et al*., 2009).

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