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Molecular Typing Reveals High Genetic Diversity of *Xanthomonas translucens* Strains Infecting Small-Grain Cereals in Iran

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ABSTRACT This study provides a phylogeographic insight into the population diversity of *Xanthomonas translucens* strains causing bacterial leaf streak disease of small-grain cereals in Iran. Among the 65 bacterial strains isolated from wheat, barley, and gramineous weeds in eight Iranian provinces, multilocus sequence analysis and typing (MLSA and MLST) of four housekeeping genes (*dnaK*, *fyuA*, *gyrB*, and *rpoD*), identified 57 strains as *X. translucens* pv. undulosa, while eight strains were identified as *X. translucens* pv. translucens. Although the pathogenicity patterns on oat and ryegrass weed species varied among the strains, all *X. translucens* pv. undulosa strains were pathogenic on barley, Harding's grass, rye (except for XtKm35) and wheat, and all *X. translucens* pv. translucens strains were pathogenic on barley and Harding's grass, while none of the latter group was pathogenic on rye or wheat (except for XtKm18). MLST using the 65 strains isolated in Iran, as well as the sequences of the four genes from 112 strains of worldwide origin retrieved from the GenBank database, revealed higher genetic diversity (i.e., haplotype frequency, haplotype diversity, and percentage of polymorphic sites) among the Iranian population of *X. translucens* than among the North American strains of the pathogen. High genetic diversity of the BLS pathogen in Iran was in congruence with the fact that the Iranian Plateau is considered the center of origin of cultivated wheat. However, further studies using larger collections of strains are warranted to precisely elucidate the global population diversity and center of origin of the pathogen.

IMPORTANCE Bacterial leaf streak (BLS) of small-grain cereals (i.e., wheat and barley) is one of the economically important diseases of gramineous crops worldwide. The disease occurs in many countries across the globe, with particular importance in regions characterized by high levels of precipitation. Two genetically distinct xanthomonads—namely, *Xanthomonas translucens* pv. undulosa and *X. translucens* pv. translucens—have been reported to cause BLS disease on small-grain cereals. As seed-borne pathogens, the causal agents are included in the A2 list of quarantine pathogens by the European and Mediterranean Plant Protection Organization (EPPO). Despite its global distribution and high economic importance, the population structure, genetic diversity, and phylogeography of *X. translucens* remain undetermined. This study, using MLSA and MLST, provides a global-scale phylogeography of *X. translucens* strains infecting small-grain cereals. Based on the diversity parameters, neutrality indices, and population structure, we observe higher genetic diversity of the BLS pathogen in Iran, which is geographically close to the center of origin of common wheat, than has so far been observed in other areas of the world, includ-

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ing North America. The results obtained in this study provide a novel insight into the genetic diversity and population structure of the BLS pathogen of small-grain cereals on a global scale.

KEYWORDS bacterial leaf streak, *Hordeum vulgare*, Iranian Plateau, MLSA, MLST, phylogeography, *Triticum aestivum*

Among the biotic constraints of small-grain cereals in the *Poaceae* family, bacterial leaf streak (BLS), caused by different pathovars of *Xanthomonas translucens* (i.e., *X. translucens* pv. translucens and *X. translucens* pv. undulosa), is considered one of the most important seed-borne pathogens around the globe (1, 2). The disease was first described on barley (*Hordeum vulgare*) in the United States in 1917, and currently, the causal agent is widespread through all six continents. *Xanthomonas translucens* is included in the A2 list of quarantine pathogens by the European and Mediterranean Plant Protection Organization (EPPO) (3). The pathogen is seed-borne, and infected seeds are the main source of inoculum for long-distance distribution. Furthermore, a humid and warm environment coupled with frequent overhead irrigation maximizes bacterial growth and plant-to-plant spread in the field, leading to 10 to 40% yield losses (2). When the symptoms are found on wheat glumes (a leaf-like structure attached to the flowers of Gramineae), the disease is called "black chaff" and is characterized by black, longitudinal, and parallel stripes (1). Yield loss due to BLS is proposed to be attributable to disease severity on the wheat spikes and flag leaf (the uppermost leaf of the plant). In severe cases of disease incidence, 5 to 10% of the wheat spikes may be sterile due to the BLS infection. It has also been noted that 50 and 100% disease severity on the flag leaf will result in 8 to 13% and 13 to 34% kernel weight losses, respectively (2).

Until recently, discrimination of *X. translucens* pathovars has mostly relied on the host ranges and symptom profiles of the pathogens on a set of gramineous species (4–6). For instance, it has been noted that *X. translucens* pv. translucens, including the strains previously designated *X. translucens* pv. hordei, is limited to barley, while *X. translucens* pv. undulosa displays a broader host range, infecting wheat, barley, oat, rye, bromes, and triticale (2, 5). Pathovar designation of *X. translucens* strains is complicated not only because of the overlapping host ranges (5, 7) but also because of similar genotypic features of the pathotype strains (3, 6). Conventional bacteriological typing techniques (e.g., repetitive element palindromic PCR [rep-PCR], amplified fragment length polymorphism [AFLP], and protein/fatty acid profiling) can only partly solve the taxonomic issues among the *X. translucens* pathovars (4, 5, 8, 9). However, it has recently been shown that multilocus sequence analysis (MLSA) and typing (MLST) using partial sequences of four housekeeping genes (*dnaK*, *fyuA*, *gyrB*, and *rpoD*) could discriminate between *X. translucens* strains isolated from gramineous crops, and the resulting scheme was in congruence with their experimental host range and host of isolation, as well as draft-genome-sequence-based phylogeny of the strains (3, 6).

Although Curland et al. (6) pinpointed the best method so far for evaluating the genetic diversity of *X. translucens* strains, the exploration was limited to North American strains, thus leaving the Old World population of the pathogen uninvestigated. Considering that the center of origin of cultivated wheat was determined to be in the Karacadağ Mountains in southeastern Turkey (10, 11), a large-scale phylogenetic analysis is warranted to shed light on the population structure and diversity parameters of *X. translucens* in the Old World and to provide a global-scale phylogeography of the species. While field symptoms resembling BLS infection have occasionally been observed for a long time on barley and wheat in Iran, the presence of *X. translucens* was scientifically first described in Kerman Province (southern Iran) in 1983 (12). However, information on the geographic distribution and phylogenetic position of the causal agent(s) is mostly lacking in the country. Given the fact that wheat is a staple food in Iran, with the highest economic importance among the agricultural commodities of the country, precise characterization and identification of the BLS pathogen will aim to

develop sustainable methods to manage the disease. Indeed, by 2016, wheat cultivation occupied over 50% of the total area dedicated to annual crops in Iran, and the areas cultivated with wheat accounted for more than 5,928,000 ha, with an annual production of 14.6 million tonnes (13). Hence, a multiphasic characterization and comparative phylogenetic analysis is warranted to decipher the population structure of the BLS pathogen in Iran, which at the same time will help to elucidate the phylogeography of cereal-infecting *X. translucens* strains at a global scale.

The objectives of the present study were to (i) assess the host range of *X. translucens* strains isolated from wheat, barley, rye, and ryegrass (*Lolium* spp.) in Iran and (ii) determine the phylogenetic positions of the strains using the 4-gene (*dnaK*, *fyuA*, *gyrB*, and *rpoD*) MLSA/MLST scheme. Furthermore, a global-scale phylogeographic analysis was conducted using the strains isolated in Iran and the publicly available sequences of 112 *X. translucens* strains of worldwide origin (6, 14). Haplotype diversity and sequence variation statistics showed that the strains isolated in Iran possess higher genetic diversity than the New World strains of the pathogen.

RESULTS

Bacterial strains and host range assays. Sixty-five Gram-negative xanthomonad-like bacterial strains were isolated from wheat, barley, and gramineous weeds in cereal-growing areas in Iran from 2008 to 2017 (Table 1). The number of surveyed fields in each area per year depended on the BLS incidence and severity reports received from the local crop sanitary inspectors. The 65 strains were isolated in eight provinces throughout southern (Bushehr, Fars, and Kerman), western (Kurdistan, Hamadan, Lorestan, and Kermanshah), and northwestern (Zanjan) Iran (Fig. S1 in the supplemental material). All of the strains formed pale-yellow colonies on yeast extract-dextrose-calcium carbonate (YDC) medium and elicited a hypersensitivity reaction (HR) on tobacco leaves. Furthermore, the specific primer pair T1/T2 (7) amplified a 139-bp DNA fragment of the $tRNA^{Ala}$ locus in all 65 strains that is useful in identifying *X. translucens* species. Consequently, all of the strains were subjected to the pathogenicity tests and host range assays.

In the host range assays, all strains were shown to be pathogenic on their host of isolation except strain XtKm15, which was isolated from ryegrass but did not induce symptoms on this species. On the other hand, all but seven strains (XtKm4, XtKm7, XtKm8, XtKm9, XtKm11, XtKm33, and XtKm34) were pathogenic on wheat, although the symptoms produced by XtKm18 on wheat plants were less severe than those produced by the other strains. All of the strains that were nonpathogenic on wheat plants had been isolated from barley. Although all of the strains were pathogenic on barley plants, the barley strain XtKm9, as well as the type strain of *X. translucens* pv. translucens (DSM 18974, used as a positive control), were pathogenic only on barley plants. All of the strains isolated from wheat were pathogenic on wheat and barley, while only half of the barley strains (7 out of 14) were pathogenic on wheat (Table 1; Fig. 1). In general, the host range of the wheat strains was broader than that of the barley strains. Twenty wheat strains, four barley strains (XtHn9, XtHn11, XtLr7, and XtLr9), and the rye strain XtKm3 were pathogenic on all six small-grain cereals tested (Fig. 1). The few remaining strains were nonpathogenic on at least one of the plant species tested under the greenhouse conditions used.

Phylogenetic analyses. Multilocus sequence analysis of four housekeeping genes (*dnaK*, *fyuA*, *gyrB*, and *rpoD*) was conducted on all 65 strains used in this study. BLAST searches using the sequences of each of the individual genes revealed that the strains isolated in Iran had 99 to 100% sequence identity with the type/reference strains of *X. translucens*, i.e., *X. translucens* pv. undulosa (LMG 892^T), *X. translucens* pv. secalis (LMG 883^T), and *X. translucens* pv. translucens (ICMP 5752^T). A phylogenetic tree constructed using the data set of concatenated sequences of *dnaK*, *fyuA*, *gyrB*, and *rpoD* genes confirmed the results obtained from the BLAST analyses. All of the strains isolated in Iran clustered in a monophyletic clade within *X. translucens*, although they were scattered through different pathovars and phylogroups of the species (Fig. 1; Fig. S2). Similar

TABLE 1 *Xanthomonas translucens* strains isolated in Iran and their host plants, geographic areas, years of isolation, and sequence types based on the partial sequences of four housekeeping genes

Strain	Host	Region		Yr	Sequence type detected in:			
		Province	County		*dnaK*	*fyuA*	*gyrB*	*rpoD*
XtLr1	Wheat	Lorestan	Borujerd	2017	8	1	6	4
XtLr2	Wheat	Lorestan	Borujerd	2017	9	1	1	4
XtLr3	Wheat	Lorestan	Borujerd	2017	1	1	6	4
XtLr4	Wheat	Lorestan	Khorramabad	2016	1	1	1	4
XtLr5	Wheat	Lorestan	Khorramabad	2016	10	1	1	5
XtLr6	Wheat	Lorestan	Khorramabad	2016	1	1	1	8
XtLr7	Barley	Lorestan	Khorramabad	2017	1	1	1	10
XtLr8	Wheat	Lorestan	Khorramabad	2016	9	1	1	6
XtLr9	Barley	Lorestan	Khorramabad	2017	1	1	1	10
XtKr1	Wheat	Kermanshah	Kermanshah	2016	8	1	6	4
XtKr2	Wheat	Kermanshah	Kermanshah	2016	9	1	1	4
XtKr3	Wheat	Kermanshah	Kermanshah	2016	1	1	3	4
XtKr4	Wheat	Kermanshah	Kermanshah	2016	11	1	1	4
XtKr5	Wheat	Kermanshah	Kermanshah	2016	1	1	1	4
XtZa1	Wheat	Zanjan	Khodabandeh	2016	8	1	6	4
XtZa2	Wheat	Zanjan	Khodabandeh	2016	9	1	1	4
XtZa3	Wheat	Zanjan	Khodabandeh	2016	8	1	1	4
XtZa4	Wheat	Zanjan	Khodabandeh	2016	8	1	1	9
XtZa5	Wheat	Zanjan	Khodabandeh	2016	1	1	1	7
XtFa1	Wheat	Fars	Firuzabad	2016	8	1	1	4
XtFa2	Wheat	Fars	Eqlid	2016	8	1	6	4
XtFa3	Wheat	Fars	Eqlid	2016	8	1	6	4
XtFa4	Wheat	Fars	Firuzabad	2016	8	1	1	10
XtFa5	Wheat	Fars	Eqlid	2016	8	1	1	4
XtFa6	Wheat	Fars	Eqlid	2016	8	1	1	4
XtFa7	Wheat	Fars	Eqlid	2016	1	1	1	4
XtKn1	Wheat	Kurdistan	Dehgolan	2016	1	1	6	4
XtKn2	Wheat	Kurdistan	Dehgolan	2016	8	1	1	4
XtKn3	Wheat	Kurdistan	Dehgolan	2016	1	1	1	4
XtKn4	Wheat	Kurdistan	Marivan	2016	1	1	1	4
XtKn5	Wheat	Kurdistan	Marivan	2016	1	1	1	4
XtHn1	Wheat	Hamadan	Nahavand	2017	8	1	1	4
XtHn2	Wheat	Hamadan	Nahavand	2017	8	1	6	4
XtHn3	Wheat	Hamadan	Nahavand	2017	1	1	1	4
XtHn4	Wheat	Hamadan	Asadabad	2016	1	1	1	4
XtHn5	Wheat	Hamadan	Asadabad	2016	1	1	1	4
XtHn6	Wheat	Hamadan	Asadabad	2016	8	1	1	4
XtHn7	Wheat	Hamadan	Asadabad	2016	12	1	1	4
XtHn8	Wheat	Hamadan	Asadabad	2016	9	1	1	4
XtHn9	Barley	Hamadan	Asadabad	2017	8	1	1	4
XtHn10	Barley	Hamadan	Asadabad	2017	1	1	1	4
XtHn11	Barley	Hamadan	Asadabad	2017	8	1	1	4
XtKm3	Rye	Kerman	Bardsir	2015	5	1	1	4
XtKm4	Barley	Kerman	Bardsir	2015	16	3	6	2
XtKm7	Barley	Kerman	Shahr-e Babak	2014	18	2	4	1
XtKm8	Barley	Kerman	Shahr-e Babak	2014	15	2	6	13
XtKm9	Barley	Kerman	Bardsir	2015	20	3	1	2
XtKm10	Barley	Kerman	Bardsir	2015	8	1	6	4
XtKm11	Barley	Kerman	Bardsir	2015	17	3	6	2
XtKm12	Wheat	Kerman	Bardsir	2015	7	1	1	4
XtKm15	Ryegrass	Kerman	Orzuiyeh	2015	1	1	6	4
XtKm18	Barley	Kerman	Baft	2015	18	2	6	13
XtKm22	Wheat	Kerman	Kerman	2008	8	1	6	4
XtKm24	Wheat	Kerman	Kerman	2008	8	1	6	4
XtKm25	Wheat	Kerman	Kerman	2015	1	1	6	4
XtKm27	Wheat	Kerman	Kerman	2015	1	1	6	4
XtKm28	Wheat	Kerman	Kerman	2009	1	1	1	4
XtKm29	Wheat	Kerman	Kerman	2015	1	1	1	4
XtKm30	Wheat	Kerman	Kerman	2015	8	1	6	4
XtKm31	Wheat	Kerman	Kerman	2015	1	1	5	4
XtKm33	Barley	Kerman	Orzuiyeh	2009	14	2	6	12
XtKm34	Barley	Kerman	Kerman	2015	13	2	6	13

(Continued on following page)

TABLE 1 (Continued)

Strain	Host	Region		Yr	Sequence type detected in:			
		Province	County		*dnaK*	*fyuA*	*gyrB*	*rpoD*
XtKm35	Wheat	Kerman	Kerman	2015	1	1	6	4
XtBu36	Wheat	Bushehr	Borazjan	2017	1	1	1	4
XtBu38	Wheat	Bushehr	Borazjan	2017	1	1	1	4
ICMP 11055	Wheat	Kerman	Kerman	1983	1	1	1	4
ICMP 5752	Barley	USA		ND[a]	21	2	1	13

[a]ND, not determined.

results were obtained when the sequences of individual housekeeping genes were subjected to the phylogenetic analysis (data not shown).

Eighty-seven percent of *X. translucens* strains isolated in Iran (57 of 65 strains) were clustered with the pathotype strains of *X. translucens* pv. undulosa and *X. translucens* pv. secalis. These strains were divided into two main subclusters (subcluster I, including 23 strains, and subcluster II, including 18 strains), as well as 16 unclustered strains (Fig. 1). Among the aforementioned 57 strains, 49 strains were isolated from wheat, 6 strains were isolated from barley, and each of the 2 remaining strains was isolated from rye or from ryegrass. According to the host of isolation, we refer to the wheat, barley and ryegrass strains as *X. translucens* pv. undulosa, while the rye strain (XtKm3) is considered *X. translucens* pv. secalis. Regardless of their geographic origin (province/county), all 49 wheat strains, as well as the 2 strains isolated from weed species (XtKm3 and XtKm15, isolated from rye and ryegrass, respectively), were identified as *X. translucens* pv. undulosa, while only 43% (6 of 14) of the barley strains were clustered in this pathovar (Fig. 1). The remaining eight barley strains were clustered among the strains of *X. translucens* pv. translucens; however, they were scattered through three subclusters. Interestingly, all of the Iranian strains identified as *X. translucens* pv. translucens were isolated from barley in Kerman Province (Fig. S3). Although strains XtKm18, XtKm33, XtKm34, XtKm8, and XtKm7 were clustered in a monophyletic group, XtKm7 was differentiated from the remaining four strains and placed in a separate subcluster (Fig. 1). Strain XtKm7 showed one and five nucleotide differences in the *gyrB* and *rpoD* gene sequences, respectively, compared to the sequences of the subcluster consisting of strains XtKm8, XtKm18, XtKm33, and XtKm34. Furthermore, strains XtKm11, XtKm4, and XtKm9, which clustered together with the strains isolated from barley in the United States, had one, three, and six nucleotide differences in the *dnaK*, *fyuA*, and *rpoD* gene sequences, respectively, from the sequences of the subgroup containing strains XtKm8, XtKm18, XtKm33, and XtKm34. Moreover, there were three nucleotides differences in the *fyuA* sequences and one nucleotide difference in the *dnaK*, *gyrB*, and *rpoD* sequences between strain XtKm7 and the three strains XtKm4, XtKm9, and XtKm11.

The phylogenetic positions of the strains were in congruence not only with their host of isolation but also with the experimental host range of the strains. For instance, all the strains identified as *X. translucens* pv. undulosa were pathogenic on wheat, barley, Harding's grass (*Phalaris aquatica*), and rye except the wheat strain XtKm35, which was not pathogenic on rye, whereas all *X. translucens* pv. translucens strains were pathogenic on barley and Harding's grass and none of them was pathogenic on wheat and rye except strain XtKm18, which induced water soaking symptoms on wheat plants. The pathogenicity patterns on ryegrass and oat plants were variable among the strains regardless of their host of isolation and phylogenetic position. Interestingly, all 23 strains that clustered in subcluster I of *X. translucens* pv. undulosa were pathogenic on all six plant species evaluated. However, neither oat nor ryegrass plants were infected with the 18 strains in subcluster II. More specifically, none of the strains XtFa2, XtFa3, XtHn2, XtKm10, XtKm22, XtKm24, XtKm30, XtKr1, XtLr1, and XtZa1, which were in the same multilocus haplotype (MH), was pathogenic on oat.

Genetic diversity. The strains isolated in Iran carried different allelic forms and sequence types (STs) and corresponded to 26 MHs based on the concatenated se-

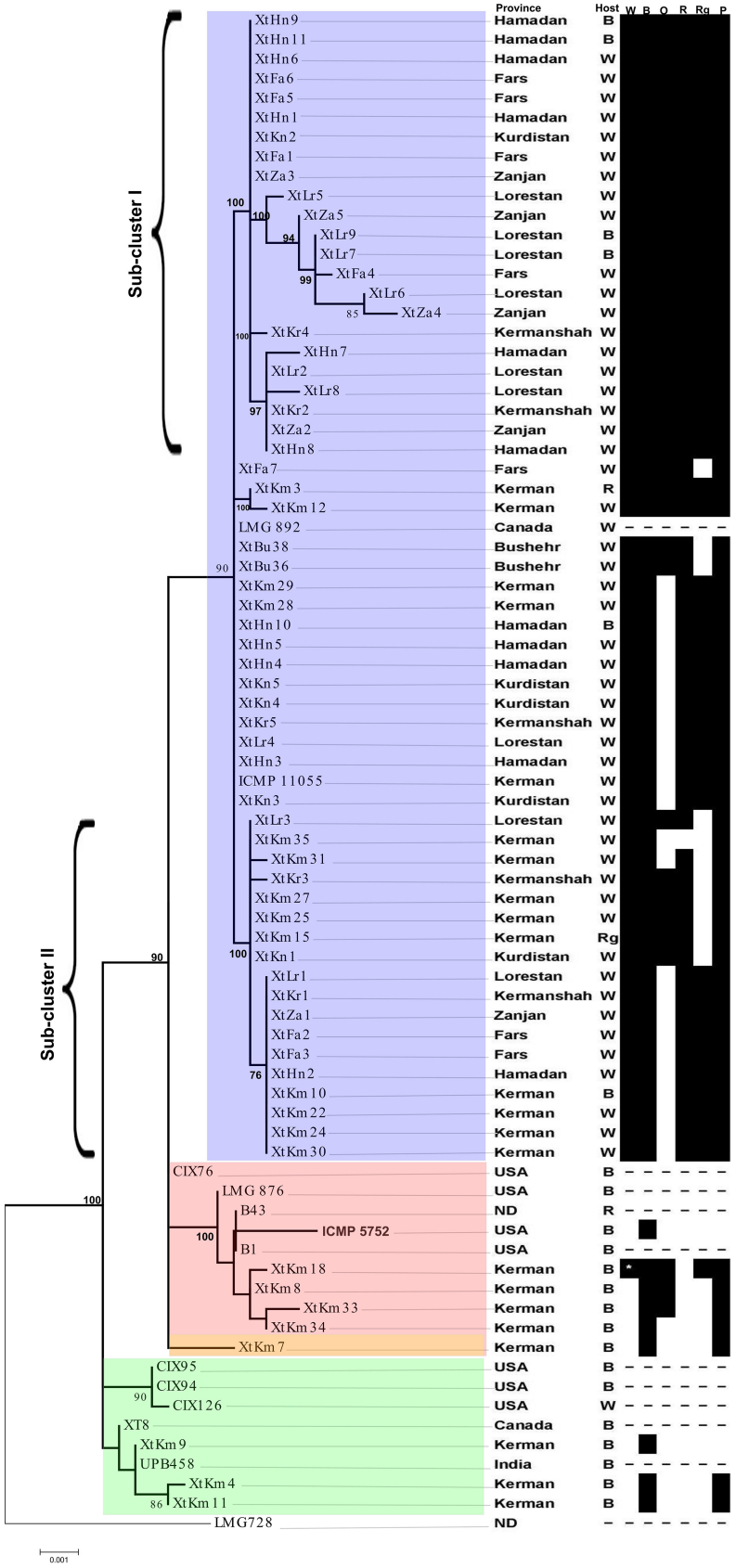

FIG 1 Phylogeny of *Xanthomonas translucens* strains isolated in Iran based on the concatenated sequences of four housekeeping genes (*dnaK*, *fyuA*, *gyrB*, and *rpoD*). *Xanthomonas translucens* pv. poae

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quences of four housekeeping genes (Table S1). As for the individual genes in the Iranian strains, 15, 3, 5, and 11 STs were detected for the *dnaK*, *fyuA*, *gyrB*, and *rpoD* genes, respectively (Fig. S3), while only 8, 3, 2, and 8 STs were detected for these genes in 112 strains from other countries (Table S1). Sequence variation statistics and diversity parameters among the strains isolated in Iran were calculated using DnaSP version 5.10 software and compared with those of 112 *X. translucens* strains isolated in six different (non-Iran) countries (Table 2). As for the 58 *X. translucens* pv. undulosa strains isolated in Iran (strains isolated in this study and the reference strain ICMP 11055 isolated in southern Iran in 1983), there were 8, 1, 4, and 7 STs in the sequences of *dnaK*, *fyuA*, *gyrB*, and *rpoD* genes, respectively, while 7, 2, 3, and 4 STs, respectively, were found in the *X. translucens* pv. translucens strains (Fig. 2). The haplotype frequency (HF; ratio of the number of haplotypes versus the number of strains) indices were 0.137, 0.017, 0.068, and 0.120 for *X. translucens* pv. undulosa and 0.875, 0.250, 0.375, and 0.500 for *X. translucens* pv. translucens strains in the *dnaK*, *fyuA*, *gyrB*, and *rpoD* genes, respectively, indicating higher genetic diversity among the *X. translucens* pv. translucens strains in Iran. Furthermore, comparison of HF and haplotype diversity (HD) indices between the strains isolated in Iran and those of 112 non-Iran strains revealed higher genetic diversity among the Iranian strains of *X. translucens* (Table 2).

Several strains isolated in different geographical areas in Iran shared the same MH with strains isolated in other countries (Fig. 2). For instance, strain XtKm3 isolated in Kerman (Iran) and strains CIX36, CIX53, and CIX54 isolated in Minnesota belonged to the same MH, while strain XtKm9 shared the same MH with barley strain UPB458 isolated in India. Interestingly, MH2 included 15 and 39 strains isolated in Iran and the United States, respectively, as well as the pathotype strains of *X. translucens* pv. undulosa (LMG 892^T) and *X. translucens* pv. secalis (LMG 883^T). On the other hand, several MHs were represented by only one Iranian strain (e.g., XtKm4, XtKm7, XtKm8, XtKm11, XtKm18, XtKm33, and XtKm34 for *X. translucens* pv. translucens and XtFa4, XtHn7, XtKr4, XtLr5, XtLr6, XtLr8, and XtZa4 for *X. translucens* pv. undulosa), which indicates a higher allelic richness of the pathogen in Iran (Fig. 2; Table S1).

Phylogenetic networks were generated using the splits-decomposition method for all the individual genes, as well as for the concatenated-sequence data set. When 178 worldwide strains were analyzed, in spite of minor reticulations in the splits-decomposition network, the pairwise homoplasy index (PHI) test did not find statistically significant evidence for recombination either in the individual genes or in concatenated data sets (data not shown). The results of the PHI test were in congruence with those of the Recombination Detection Program (RDP), which did not find statistically significant evidence for recombination. On the other hand, none of the population neutrality indices (i.e., Tajima's *D*, Fu and Li's *D**, and Fu and Li's *F**) was significant among the 66 strains isolated in Iran, thus indicating the lack of evidence for selection among the population and that the population is evolving per mutation-drift equilibrium (Table 2). On the other hand, the non-Iran *X. translucens* pv. undulosa strains showed significant departure from the mutation-drift equilibrium in their *rpoD* gene sequences. The negative value of neutrality indices in *rpoD* gene sequences of non-Iran *X. translucens* pv. undulosa strains suggests a recent selective sweep or/and population expansion

FIG 1 Legend (Continued)
(LMG 728^T) was used as an out-group to root the tree. The bar presents numbers of substitutions per site. Among the 65 Iranian strains, 57 strains were identified as *X. translucens* pv. undulosa, while 8 strains were identified as *X. translucens* pv. translucens. The blue background indicates *X. translucens* pv. undulosa strains, while red, yellow, and green backgrounds indicate different subclusters of *X. translucens* pv. translucens. The black-and-white table at the right shows hosts of isolation (Host) and host ranges of the strains on six gramineous species, including wheat (W), barley (B), oat (O), rye (R), ryegrass (Rg), and Harding's grass (P [*Phalaris*]), where black squares indicate pathogenicity and white squares indicate nonpathogenicity. A dash mark in the host range table means that pathogenicity has not been determined. The atypical strain XtKm18 (indicated with an asterisk inside a black square), which was isolated from barley and identified as *X. translucens* pv. translucens, induced water-soaking symptoms on wheat plants.

TABLE 2 Sequence variation statistics of the partial sequences of four housekeeping genes from *Xanthomonas translucens* strains isolated in Iran in comparison to those of the strains isolated in six other countries

Region, *X. translucens* pathovar	Gene	No. of			Haplotype frequency[a]	Total no. of segregating sites	% of polymorphic sites	Nucleotide diversity (π)	No. of mutations (η)	Haplotype (gene) diversity	Value for indicated neutrality test			Minimum no. of recombination events
		Strains	Nucleotides	Haplotypes							Tajima's *D*	Fu and Li's *D**	Fu and Li's *F**	
Iran														
undulosa	*dnaK*	58[b]	762	8	0.137	5	0.656	0.118×10^{-2}	5	0.655	−0.391 NS[c]	1.083 NS	0.724 NS	2
	fyuA	58	522	1	0.017	0	0.000	0.000	0	0.000	NP[d]	NP	NP	0
	gyrB	58	505	4	0.068	3	0.594	0.100×10^{-2}	3	0.456	−0.442 NS	−1.782 NS	−1.602 NS	0
	rpoD	58	674	7	0.120	6	0.890	0.123×10^{-2}	7	0.257	−1.165 NS	−0.369 NS	−0.737 NS	2
translucens	*dnaK*	8	762	7	0.875	4	0.524	0.230×10^{-2}	4	0.964	0.586 NS	0.568 NS	0.629 NS	1
	fyuA	8	522	2	0.250	3	0.574	0.308×10^{-2}	3	0.536	1.600 NS	1.233 NS	1.445 NS	0
	gyrB	8	505	3	0.375	2	0.396	0.099×10^{-2}	2	0.464	−1.310 NS	−1.409 NS	−1.513 NS	0
	rpoD	8	674	4	0.500	7	1.038	0.540×10^{-2}	7	0.786	1.662 NS	0.973 NS	1.252 NS	0
Non-Iran														
undulosa	*dnaK*	75	762	8	0.106	8	1.049	0.129×10^{-2}	8	0.598	−1.011 NS	0.512 NS	0.004 NS	1
	fyuA	75	522	2	0.026	1	0.191	0.005×10^{-2}	1	0.027	−1.062 NS	−1.947 NS	−1.958 NS	0
	gyrB	75	505	2	0.026	1	0.198	0.010×10^{-2}	1	0.053	−0.904 NS	0.513 NS	0.110 NS	0
	rpoD	74[e]	674	5	0.067	5	0.741	0.031×10^{-2}	5	0.155	−1.780 NS	−3.024[e] ($P < 0.05$)	−3.084[e] ($P < 0.05$)	0
translucens	*dnaK*	36[e]	762	2	0.055	1	0.131	0.042×10^{-2}	1	0.322	0.495 NS	0.574 NS	0.636 NS	0
	fyuA	37	522	2	0.054	3	0.574	0.200×10^{-2}	3	0.348	1.009 NS	0.924 NS	1.102 NS	0
	gyrB	37	505	1	0.027	0	0.000	0.000	0	0.000	NP	NP	NP	NP
	rpoD	37	674	5	0.135	6	0.890	0.353×10^{-2}	6	0.701	1.777 NS	0.386 NS	0.949 NS	0

[a]Number of haplotypes/number of strains.
[b]Fifty-seven strains isolated in this study and the reference strain ICMP 11055, isolated in southern Iran in 1983.
[c]NS, not significant.
[d]NP, no polymorphism.
[e]The *dnaK* and *rpoD* gene sequences in strains ICMP 5752 and CIX127, respectively, were excluded from the analyses due to unsatisfactory sequence quality.

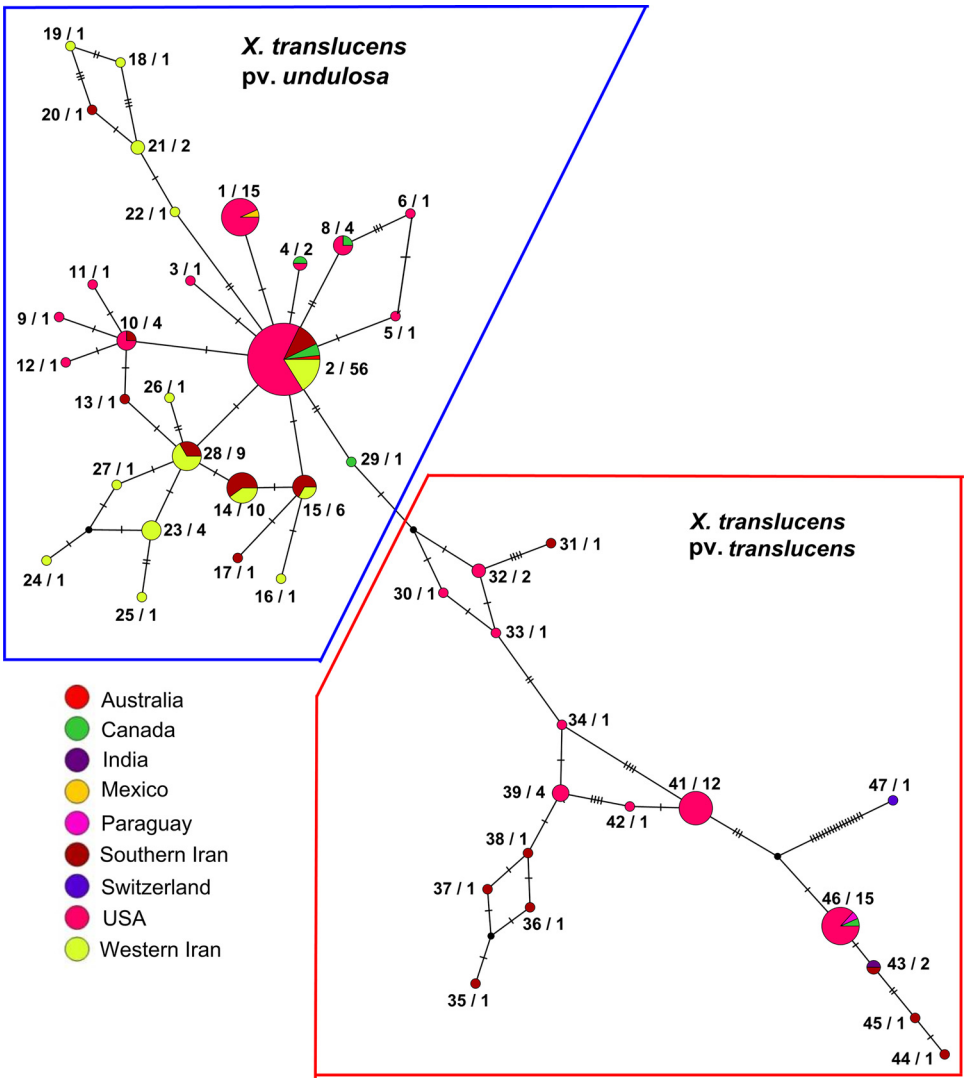

FIG 2 TCS multilocus haplotype (MH) network generated using the POPART program from the concatenated partial sequences of four housekeeping genes (*dnaK*, *fyuA*, *gyrB*, and *rpoD*) in 178 *Xanthomonas translucens* strains from Iran and seven countries around the globe. The size of a circle indicates the relative frequency of sequences belonging to a particular MH. Hatch marks along the branches indicate the numbers of mutations. Each color indicates a different geographic area. To provide precise insight into the diversity of the pathogen, strains isolated in southern Iran and western Iran were considered distinct groups. The number to the left of a slash represents the MH number (Table S1), while the number to the right of a slash indicates the number of strains in a given MH. *Xanthomonas translucens* pv. undulosa strains are surrounded by blue lines, while *X. translucens* pv. translucens strains are surrounded by red lines. MH2, consisting of 56 *X. translucens* pv. undulosa strains, was considered the founder genotype of the pathovar as confirmed by the phylogeographic analyses using the eBURST program (Fig. S5).

after a recent bottleneck, although further investigations using the sequences of additional housekeeping genes are needed to prove these observations.

Phylogeography of strains of worldwide origin. Several lines of evidence of geographic structure in the ST distribution were detected in the networks built by the TCS algorithm implemented in POPART version 1.7 (Fig. 3). Although a number of STs were shared among the strains of worldwide origin, country-specific STs were more prevalent in the *dnaK* and *rpoD* networks. For instance, in the *dnaK*, *gyrB*, and *rpoD* sequences, there were 11, 4, and 7 STs consisting of Iranian strains, while only 2 and 4 STs in the *dnaK* and *rpoD* sequences, respectively, corresponded to those in United States strains (Fig. 3). On the other hand, strain DAR61454 isolated in Australia shared the same MH with the strains isolated in Iran, Canada, and the United States, while strain UPB458

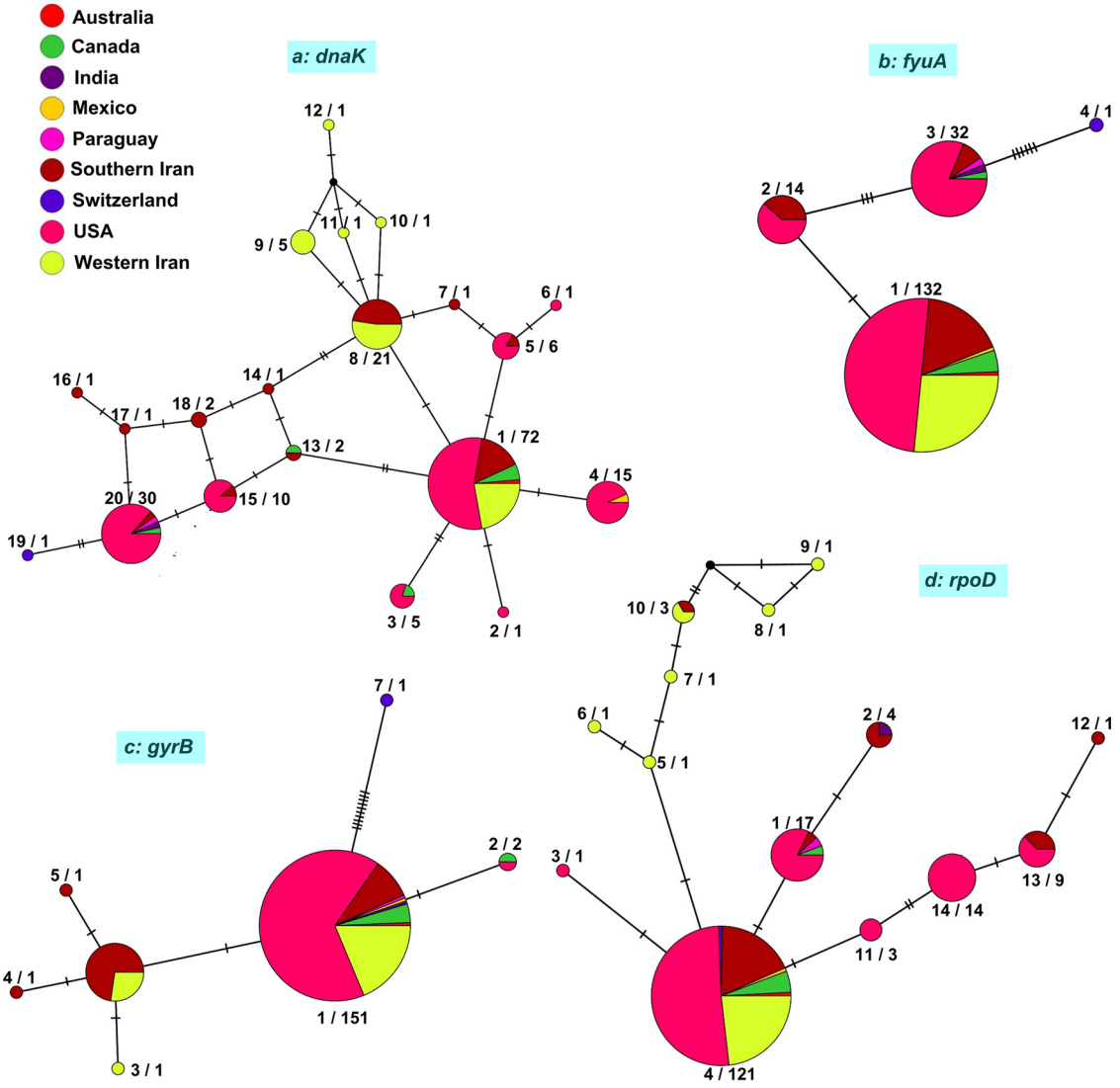

FIG 3 TCS haplotype network generated using the POPART program from the partial sequences of four housekeeping genes (*dnaK*, *fyuA*, *gyrB*, and *rpoD*) in 178 *Xanthomonas translucens* strains. The size of a circle indicates the relative frequency of sequences belonging to a particular sequence type. Hatch marks along the branches indicate the numbers of mutations. Each color indicates a different geographic area. To provide precise insight into the diversity of the pathogen, strains isolated in southern Iran and western Iran were considered distinct groups. The number to the left of a slash represents the ST number (Table S1), while the number to the right of a slash indicates the number of strains in a given ST. Strains isolated in Iran generated several unique haplotypes, indicating a higher genetic diversity of the pathogen in Iran.

isolated in India shared the same MH with strains isolated in southern Iran (Fig. 2; Table S1), raising the speculation that strain UPB458 originated in Iran. Furthermore, six strains isolated in Canada (XT5523, XT5770, XT5791, XT8, LMG 883, and LMG 892), as well as strains UPB513 and UPB787 isolated in Mexico and Paraguay, respectively, shared the same MH with strains isolated in the Midwestern United States (Fig. S4). The patterns of MH positioning in the TCS network (Fig. 2 and 3) were in congruence with the MrBayes bifurcating trees; however, reticulations in the TCS networks of the *dnaK* and *rpoD* sequences, as well as of the concatenated sequences, suggest a more complex evolutionary history of *X. translucens* than can be observed in the phylogenetic trees (Fig. 1).

Using the eBURST analysis, 178 *X. translucens* strains were assigned to 46 MHs within three groups and four singleton sequences. Group one consisted of 143 strains and 37 MHs (Fig. S5), while groups two and three, both consisting of only *X. translucens* pv. translucens strains, included 30 and 2 strains, as well as 4 and 2 MHs, respectively (data not shown). Although the founder genotype of *X. translucens* pv. undulosa (MH2) and

the respective subgroups in group one were supported with 99 to 100% bootstrap values (Fig. S5), assignment of the founder genotype in groups two and three was not supported by high bootstrap value (>20), and hence, they were not considered reliable founder genotypes. The founder genotype MH2 included 56 strains (as shown in Fig. 2), with 20, 9, and 4 single-, double-, and triple-locus variants, respectively. Two single-locus variants of MH2 (MH10 and MH28) were identified as the subgroup founders, each with its own cluster of linked single-locus variants (Fig. S5). Among the 56 strains in MH2, 15 strains were isolated in Iran, while 40 and 1 strain were isolated in the United States and Australia, respectively. The results obtained with eBURST analyses were in congruence with those of POPART analyses, suggesting MH2 as the primary founder genotype for *X. translucens* pv. undulosa (Fig. 2; Fig. S5).

DISCUSSION

This study describes the host range and phylogenetic positions of 65 *X. translucens* strains isolated from gramineous plants in Iran among those of the worldwide population of the pathogen. All the wheat strains isolated in Iran were identified as *X. translucens* pv. undulosa, while the strains isolated from barley were shown to belong to two pathovars (*X. translucens* pv. undulosa and *X. translucens* pv. translucens). Only one (XtKm18) of the *X. translucens* pv. translucens strains isolated in Iran was pathogenic on wheat, while all of the strains were pathogenic on barley and Harding's grass. The relatively higher number of wheat strains (49/65) isolated in our surveys might be attributed to the differences in the cultivated areas covered by the two crops and the proportionally higher number of wheat fields surveyed in comparison to the number of barley fields. Similar results in terms of multispecies etiology and variable host range were observed in the bacterial agents infecting solanaceous vegetables (15), edible dry beans (16), and sugar beets (17) in Iran.

As revealed by several diversity parameters (haplotype frequency, haplotype diversity, and percentage of polymorphic sites), the strains isolated in Iran possess significantly higher genetic diversity than the strains isolated in the North American countries. Furthermore, a global-scale phylogeographic analysis supports the hypothesis that Iranian strains of *X. translucens* have acted as the founder population in the other countries (Fig. S4 and S5 in the supplemental material). Several unique MHs were determined among the Iranian strains, highlighting the allelic richness of the *X. translucens* population in Iran. All these lines of evidence support the hypothesis that a given plant pathogen possesses higher genetic diversity in the center of origin of its host plant, which is the case for the BLS pathogen. The results of neutrality tests (Fu and Li's $D^*$and Fu and Li's F^*) on the *rpoD* gene sequences of non-Iran *X. translucens* pv. undulosa strains also support the hypothesis, suggesting a selective sweep and population expansion after a recent bottleneck in North America, while additional housekeeping gene sequences need to be examined to further validate the hypothesis (Table 2). Since the center of origin of cultivated wheat was determined to be in southeastern Turkey (10, 11), we speculate that *X. translucens* should be an endemic pathogen in the Middle Eastern countries, including Iran. Unlike modern crops, such as tomato (*Solanum lycopersicum*), which have only a short history of cultivation and trade on the global scale, dating to the early 16th century (18), the history of the worldwide distribution of common wheat by humankind is not clear, making the phylogeographic study of cereal pathogens complicated. BLS was reported in the neighbor countries of Iran, and hence, a comprehensive epidemiological investigation will decipher the distribution, population structure, and genetic diversity of *X. translucens* in the center of origin of cultivated wheat.

According to the eBURST and POPART analyses, MH2—including 56 of 178 strains—was determined to be the founder genotype of *X. translucens* pv. undulosa (Fig. S5). Interestingly, the pathotype strains of *X. translucens* pv. undulosa (LMG 892^T) and *X. translucens* pv. secalis (LMG 883^T) (both were isolated in southern Canada in 1966) belong to MH2 (Table S1). The pathotype strain of *X. translucens* pv. undulosa was isolated from *Triticum turgidum*, while the pathotype strain of *X. translucens* pv. secalis was isolated from rye (6). It is likely that the two pathotypes originated from the same

founding genotype in the North America; however, the prevalence of the "one host-one species" concept in the mid-20th century led to the designation of the two strains as two different taxa at that time (4). *Triticum turgidum*, from which the pathotype strain of *X. translucens* pv. undulosa was isolated, is also known as Khorasan wheat (19). The common name refers to Khorasan Province in northeastern Iran and indicates the origin of the crop in the ancient civilizations in the Iranian Plateau or Fertile Crescent (19). Khorasan wheat was accidentally introduced into the United States in 1949 and planted for the first time in Fort Benton, MT, while extensive cultivation of the crop in the United States only started in 1964 (19). Since the founding genotype of the pathogen is still prevalent in the Iranian wheat cultivation areas, one can hypothesize that *X. translucens* pv. undulosa was transmitted from the Iranian Plateau into the United States via infected seeds of Khorasan wheat in the mid-20th century. All these lines of evidence bring us to the speculation that the existing population of *X. translucens* pv. undulosa in North America was introduced into the New World from the Iranian Plateau and dispersed throughout the continent during the past 50 years (Fig. S4) (6). However, further genotypic and phylogeographic examinations using a broader set of strains are needed to validate this hypothesis.

In conclusion, the results of the present study revealed a greater diversity among the worldwide population of *X. translucens* than had been described before. More specifically, based on the phylogeographic analyses, we have noted that the strains isolated in the Iranian Plateau—which is considered the center of origin of cultivated wheat—were genetically more diverse than the strains isolated in the New World. These results suggest that the phylogeny of wheat- and barley-pathogenic members of *X. translucens* should be reexamined using a larger collection of strains from all the known hosts of the pathogen, including gramineous weeds, from all over the world. Moreover, despite a few preliminary studies (20–23), these findings raise questions about whether there is any source of resistance among the wild population of wheat species (*Triticum* spp.) in the center of origin of this crop and emphasize at the same time the need for a more detailed investigation in this regard. Only future studies, based on population genetics, comparative genomics, and pathogenicity assays of a wide collection of strains isolated from different geographical regions, can shed more light on these questions.

MATERIALS AND METHODS

Bacterial strains and their identification. The bacterial strains used in this study were obtained during a decade-long (2008 to 2017) series of field surveys across cereal-growing areas of northwestern, western, central, and southern Iran. Furthermore, seed lots of small-grain cereals deposited in the seed banks of the Seed and Plant Certification and Registration Institute (Karaj, Iran) were investigated for BLS infestation. The surveying strategy and sampling procedure were the same as described previously (24, 25). Bacterial strains were isolated from the plant tissues and seed samples using the methods described previously (26) with minor modifications (27). All of the strains (Table 1) were subjected to preliminary phenotypic tests (26). Gram reaction and colony characteristics on yeast extract-dextrose-calcium carbonate (YDC) agar medium were determined. The hypersensitivity reaction (HR) was evaluated on tobacco (*Nicotiana tabacum* cv. Turkish) leaves, using the bacterial suspension from a 48-h culture on NA medium (26) at a concentration of 10^8 CFU/ml. Reference strains of *X. translucens* pv. undulosa (ICMP 11055) and *X. translucens* pv. translucens (DSM 18974^T) were included as controls in all of the tests. Phenotypic tests were repeated twice.

Pathogenicity tests and host range assays. All the bacterial strains (Table 1) were evaluated for their pathogenicity on six gramineous species, including their host of isolation, as well as common gramineous weeds in the region. Barley (cv. Valfajr), Harding's grass, oat, rye, ryegrass, and wheat (cv. Pishtaz) plants were inoculated with the bacterial suspensions using a procedure described previously (27). The inoculated plants were maintained under greenhouse conditions with 90 to 95% relative humidity and periodically (daily) monitored for symptom development until 25 days postinoculation (dpi). The same numbers of plants were treated with the reference strains of *X. translucens* pv. translucens (DSM 18974^T) and *X. translucens* pv. undulosa (ICMP 11055) as positive controls. Negative-control plants were treated in the same manner except that sterile distilled water was used instead of the bacterial suspensions. Koch's postulates were accomplished by reisolating the inoculated strains from plants showing symptoms on YDC medium. Confirmation of the reisolated bacteria was made using *X. translucens*-specific PCR primers T1/T2 (Table 3) (7). The pathogenicity tests were repeated twice.

Molecular phylogenetic analysis. All strains showing xanthomonad-like characteristics (i.e., pale-yellow mucoidal colonies on YDC medium and hypersensitivity reaction on tobacco leaves) were subjected to the diagnostic PCR test using the *X. translucens*-specific primer pair T1/T2 (Table 3) (7). The bacterial DNA extraction and PCR procedures were described previously (27); the primer sequences and

TABLE 3 Primer pairs used in this study

Primer name	Nucleotide sequence (5′–3′)	Size of amplicon (bp)	Annealing temp (°C)	Target region	Reference
T1	CCGCCATAGGGCGGAGCACCCCGAT	139	53	16S-23S rRNA	7
T2	GCAGGTGCGACGTTTGCAGAGGGATCTTCTGCAAA				
XrpoD1F	TGGAACAGGGCTATCTGACC	674	54	*rpoD*	37
XrpoD1R	CATTCYAGGTTGGTCTGRTT				
dnaK-F	TCCTAAGCACCTCAACATCAAG	762	59	*dnaK*	6
dnaK-R	CTTCTTGTCGTCCTTGACCTC				
fyuA-F	CTCGCAGAACGGCCTGTA	522	61	*fyuA*	6
fyuA-R	GTAGCCGGGCATCTTCAACT				
XgyrPCR2F	AAGCAGGGCAAGAGCGAGCTGTA	700	54	*gyrB*	38
X.gyrrsp1	CAAGGTGCTGAAGATCTGGTC				

annealing temperatures are provided in Table 3. Sixty-five *X. translucens* strains were identified using the species-specific PCR primer and were subjected to MLSA/MLST analyses using the sequences of four housekeeping genes (*dnaK*, *fyuA*, *gyrB*, and *rpoD*), as recommended previously (6).

To determine the phylogenetic positions of the strains isolated in Iran and access a global-scale phylogeography for the BLS pathogen, sequences of the four housekeeping genes in a collection of 112 worldwide *X. translucens* strains were retrieved from the GenBank database and included in the phylogenetic analyses (Table S1 in the supplemental material) (6, 14). The sequences were concatenated following the alphabetic order of the genes, resulting in a sequence of 2,464 bp, as follows: nucleotides 1 to 763 for *dnaK*, 764 to 1285 for *fyuA* (522 bp), 1286 to 1790 for *gyrB* (505 bp), and 1791 to 2464 for *rpoD* (674 bp) genes. Phylogenetic trees were constructed using MrBayes 3.1.0, running the chain for 1×10^6 generations (bootstrapping at 1,000 replications) and setting the burn-in at 1,000 (28). The substitution model for Bayesian analysis was determined, using MrModelTest version 2.3 software, to be AIC+[GTR+I] (29). The resulting phylogenetic trees were annotated and presented using MEGA 6.06 software (30).

Genetic diversity and recombination analyses. Nucleotide diversity, numbers of STs and MHs, haplotype diversity, haplotype frequency, percentages of polymorphic sites, minimum numbers of recombination events, and the ratio of the rate of nonsynonymous to synonymous mutations were estimated using DnaSP 5.10 software (31). The class I neutrality tests (Tajima's *D*, Fu and Li's *D**, and Fu and Li's *F** statistics) were also calculated for detecting departure from the mutation-drift equilibrium (31). Detection of potential recombinant sequences and identification of likely parental sequences were carried out using a set of seven nonparametric detection methods (RDP, Geneconv, MaxChi, Chimera, BootScan, SiScan, and 3Seq) implemented in Recombination Detection Program (RDP) version 4.80 (32). The analysis was performed with the default settings for the different detection methods, and the Bonferroni-corrected *P* value cutoff was set at 0.05. Recombination events were accepted when they were identified by at least four of the seven detection methods (32). A splits-decomposition network was constructed, and the pairwise homoplasy index (PHI) was calculated using SplitsTree version 4.14.4 (33). These calculations were performed once for 66 Iranian strains and again for the entire data set (178 strains) for all the individual genes, as well as for the concatenated sequences (33).

Phylogeographic analyses. To visualize the phylogeographic relationships between the strains, haplotype networks were inferred for individual housekeeping genes using the TCS algorithm (34) implemented in POPART version 1.7 (35). The geographic distribution of 178 strains collected in seven countries (Australia, Canada, India, Iran, Paraguay, Mexico, and the United States) was employed in building the haplotype network of each of the four housekeeping genes, as well as concatenated sequences as described by Leigh and Bryant (35). The country-specific STs, as well as the shared MHs between the countries, were estimated based on the resulting haplotype networks (Table S1). Furthermore, a global-scale geographical structure of *X. translucens* was visualized using the eBURST software (36). The eBURST analysis was used to explore the patterns of evolutionary descent among the strains used in this study as described by Feil et al. (36). The analyses were performed using the stringent (default) group definition, in which sequence types are included within the same clonal complex only if they share identical alleles at three or four of the four MLST loci with at least one other ST in the population. Furthermore, as a default setting, 1,000 resamplings were performed for bootstrapping the resultant evolutionary network.

Accession number(s). The nucleotide sequences of the strains isolated in Iran were deposited in the GenBank database with the following accession numbers: MK622011 to MK622075 for *dnaK*, MK622076 to MK622140 for *fyuA*, MK622141 to MK622205 for *gyrB*, and MK622206 to MK622270 for *rpoD* sequences. The GenBank accession numbers for the 112 strains of worldwide origin are listed in Table S1 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01518-19.

SUPPLEMENTAL FILE 1, PDF file, 2.5 MB.

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E.O. and M.K. conceived and designed the study, with assistance from S.M.T., H.H., P.K., and R.K. M.K. carried out the experiments. E.O. analyzed and interpreted the data with assistance from M.K., H.H., R.K., and C.B. M.K. and E.O. prepared the paper, with assistance from C.B., R.K., and G.C. E.O. revised the final manuscript and acted as the corresponding author.

The authors declare no competing financial interests.

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