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# INVESTIGATION OF RELATEDNESS BETWEEN THE GENETIC DIVERSITY AND DISEASE SEVERITY OF *ERWINIA AMYLOVORA* STRAINS OBTAINING FROM PEARS IN TURKEY

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**ABSTRACT**

*Erwinia amylovora*, which causes fire blight disease, is one of the biggest threats to pome fruit growing worldwide and is one of the top ten most destructive bacterial pathogens. In this study, the relationships between the genetic diversity and disease severity of *E. amylovora* strains isolated from pears from different regions of Turkey were revealed by pEA29 plasmid DNA derived from A/B primers, amsG primers responsible for amylovoran production, CRISPR 1, 2 and 3 primers, used to characterize the genetic diversity and some properties of the pathogen. The findings indicated significant regional differences in percent disease severity and indices among pear strains. However, no genetic variation was observed in pEA29 and amylovoran production. Only one strain differed from the others with CRISPR 2 primers, and it was observed that the specific primers used to determine the relationship between regional differences and virulence of the strains were not completely sufficient to reveal the genetic difference. The results of the study indicated that *E. amylovora* is subject to changing ecological and climatic factors in different hosts, in different countries and regions. This scenario may have induced genetic heterogeneity among the examined strains, implying that variations in virulence could also emerge in strains harboring distinct plasmid DNAs. New perspectives can be developed in the control of the disease with a full understanding of the relationships between the pathogen's pathogenicity levels and genetic structure.

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## INTRODUCTION

The global production of pome fruits averages approximately 113.750.265 tons annually (FAO, 2017). Turkey occupies a commendable third position, trailing behind China and the United States, with a production volume of 5,215,625 tons, predominantly comprising apples, pears, and quinces (FAO, 2025).

In pear (*Pyrus communis* L.) cultivation, China leads with an impressive output of 17 million tons, while Turkey ranks fourth, with 531 thousand tons, following the United States and Argentina, and is cultivated across various regions (TÜİK, 2025). Numerous diseases adversely impact pear production, leading to significant declines in both quantity and quality. *Erwinia amylovora*, the first and most damaging bacterial agent identified in plants, causes fire blight in about 140 plant species, mainly affecting members of the Rosaceae family, such as apple, pear, quince, loquat, and ornamental plants

(Vanneste, 2000; Van der Zwet, 2006).

*E. amylovora* represents the most critical and destructive affliction of pear trees on a global scale. No known pear species exhibit complete resistance to this pathogen, which is also listed on the EPPO A2 quarantine list (EPPO, 2024). The typical symptoms of fire blight in pears manifest as necrosis on flowers, leaves, fruits, branches, and shoots; the development of shepherd's crook symptoms on young shoots; fruit mummification; and the presence of bacterial exudates (ooze) (Van der Zwet and Keil, 1979; EPPO, 2024).

The economic data of fire blight disease are substantial, resulting in significant losses worldwide. The records indicate that this bacterium has instigated major epidemics, leading to financial losses in the millions of dollars (Bonn *et al*., 2000; Norelli *et al*., 2003). In Turkey, the first instance of fire blight was documented in 1998 within pear trees in the Sultandağı district of Afyonkarahisar Province, with subsequent spread identified in numerous pear orchards nationwide, inflicting considerable damage (Momol and Yegen, 1993). Biochemical, morphological, and physiological investigations have revealed a reported homogeneity among *E. amylovora* strains. Various molecular techniques have been employed in numerous studies to elucidate the genetic diversity and dissemination of this pathogen at the strain level, facilitating ecological, epidemiological, and evolutionary predictions concerning fire blight's spread in specific geographic areas (McManus and Jones, 1995; Rico *et al*., 2004; McGhee *et al*., 2011; Ivanovic *et al*., 2012; Pulawska and Sobiczewski, 2012; Krivokapic *et al*., 2018; Parcey *et al*., 2020; Popovic *et al*., 2020; Song *et al*., 2021). Additionally, the CRISPR technique has been utilized as a novel molecular method to determine the genetic diversity and certain characteristics of *E. amylovora* strains (McGhee and Sundin 2012; Parcey *et al*., 2020; Kurz *et al*., 2021; Parcey *et al*., 2022; Ibrahim *et al*., 2024; Öztürk and Kayaarslan, 2024).

This study endeavors to explore the interactions between the virulence levels and genetic variances of *E. amylovora* strains procured from diverse regions of commercial pear cultivation, with the overarching aim of predicting ecological structures and epidemiological losses at both province and regional levels.

## MATERIALS AND METHODS

### Plant Sample Collection and Bacterial Isolation and Identification

In the years 2021-2022, samples of shoots, branches, leaves, and fruits were collected from pear trees showing symptoms of fire blight in the provinces of Antalya and Kahramanmaraş (Mediterranean Region), Konya and Nevşehir (Central Anatolia Region), Bursa (Marmara Region), Afyonkarahisar (Aegean/Western Anatolia Region), Iğdır (Eastern Anatolia Region), and Samsun (Black Sea Region) during the late spring and summer periods (Figure 1).


Figure 1. Pear orchards infected with *Erwinia amylovora* (a and b) and blighted symptoms on plant materials (c: shoot, d: peduncle and flower).

Plant samples showing fire blight symptoms were washed with tap water. Small plant pieces (about 1 g) were disinfected in 1.5% sodium hypochlorite for 2 minutes, rinsed three times with sterile distilled water, and dried on

sterile blotting paper. These pieces were soaked in 50 ml of sterile phosphate buffered saline (PBS) at 25 °C for 30 min and then dilutions of up to $10^{-3}$ were prepared. Approximately 50 µl of diluted suspensions were spread on three different media such as NA, NSA, and King's B in Petri dishes: Bacterial suspensions were spread in Petri dishes and incubated at 25 °C for 36-48 h. Colonies on King's B were not fluorescent under UV light at 366 nm after 48 h. After bacterial colonies developed, those showing morphological features compatible with *E. amylovora* were purified on King's B medium (Taylor *et al*., 2001). Single colonies exhibiting typical morphological development were selected, and the purified strains were preserved in Nutrient Broth with a 30% glycerol solution at -80 °C in a deep freezer until used in experiments.

**Biochemical and Physiological Tests**

All isolated bacterial strains were identified based on various tests, including Gram staining, growth at 36 °C, levan production, fluorescent pigment formation on King's B medium, oxidative/fermentative reactions, oxidase, esculin and arginine dihydrolase tests, pectolytic activity, gelatin liquefaction and acid production from sucrose, erythritol, sorbitol, mannitol, and inositol (Lelliott and Stead 1987; Schaad *et al*., 2001). Each test was conducted three times for each strain. The EA29 reference strain, obtained from the Molecular Bacteriology Laboratory of Selcuk University Faculty of Agriculture Dept. of plant Protection, was used as a positive control in all tests.

**Hypersensitivity Reaction on Tobacco, Pathogenicity Test on Pear Seedlings and Immature Pear Fruitlets**

Pathogenicity and hypersensitivity reaction (HR) tests were carried out on young shoots of 3-year-old healthy Santa Maria variety pear seedlings grafted onto Mahaleb rootstock and on 8-week-old tobacco plants (*Nicotiana tobaccum* var. White Burley) according to Aldwinckle *et al*. (2001) and Bonasera *et al*. (2006), respectively. For pathogenicity tests, shoot inoculation and HR test on immature pear slices, all stock pear isolates at -80 C were grown on Nutrient Agar (NA) medium for 48 h and bacterial suspensions were prepared at a density of $10^8$ CFU $ml^{-1}$ (OD:600, 0.15, in Eppendorph Biophotometer) and inoculated into the plants. Using sterile scissors dipped into the prepared suspension, the tips of the young leaves of the pear seedlings were cut and they were immersed in the suspension for 30 s (Bonasera *et al*., 2006). The suspensions were injected between the leaf veins of tobacco plants using a 0.46 mm diameter (26 gauge) hypodermic syringe (Klement and Goodman, 1967). Three replicates were made for each isolate. The treated shoots were labeled with isolate codes for evaluation (Norelli *et al*., 1984). In addition, control plants were inoculated with sterile distilled water using the same method. All pear seedlings were additionally misted immediately after inoculation and covered with a polyethylene bag for 24 h to maintain high humidity. Plants were kept in greenhouse conditions at 16/8 hours photoperiod and 24±2 °C for disease development. For the test on immature pear fruits, bacterial isolates were grown in Luria Bertani (LB) broth for 36 h and sliced into thin slices, obtaining from pears in Turkey and placed in humidified petri dishes. Inoculated fruits with *E. amylovora* suspension density in $10^8$ CFU $ml^{-1}$ were incubated for 3 days at 28 °C (Van der Zwet, 1986).

**Molecular Identification of the Bacterial Strains**

**Isolation of genomic DNA and PCR amplification**

Single colonies isolated from fresh cultures grown on NA medium for 24-48 hours were transferred into tubes containing 5 ml of Luria-Bertani (LB, Merck, Germany) medium and incubated with shaking at 140 rpm for 48 hours. Genomic DNA was extracted from the pellet of cells obtained from 1 ml of liquid culture using the Bacterial DNA Isolation Kit (Qiagen, Germany), following the manufacturer's instructions. To assess the quantity and purity of the isolated DNA, spectrophotometric measurements were conducted with a Thermo Scientific Nanodrop 2000 (USA), and the DNA samples were subsequently stored at -30 °C.

The final volume of the PCR reactions was prepared to a total volume of 25 µl, consisting of 12.5 µl Master Mix (Thermo Fisher Scientific), 2 µl of forward and reverse primers from each one, 0.5 µl of $MgCl_2$, 2 µl of bacterial DNA, and 6 µl of sterile distilled water. The PCR reactions were carried out in a thermal cycler (Personal, Eppendorf) with the A/B primers using the following protocol: 95 °C for 3 min (one cycle), followed by 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min (35 cycles), with a final extension at 72 °C for 10 min (Bereswill *et al*., 1992). For the amsG-F and amsG-R primers responsible for amylovoran production, the PCR protocol was as follows: 95 °C for 10 min (one cycle), then 94 °C for 15 sec, 62 °C for 25 sec, and 72 °C for 25 sec (45 cycles), with a final extension at 72 °C for 10 min (Pester *et al*., 2012).

**Determination of Genetic Diversity in Pear Strains**

The genetic diversity among *E. amylovora* pear strains was analyzed using CRISPR-PCR. The PCR reactions were

carried out in a total volume of 25 µL, consisting of 12.5 µL of 2X PCR Master Mix, 1 µL of each primer, 8.5 µL of sterile distilled water, and 2 µL of target DNA. The PCR protocol for CRISPR 1 primers was conducted as follows: 94 °C for 5 min (1 cycle), then 94 °C for 30 sec, 58 °C for 30 sec (for CRISPR 1 and 2), or 55 °C (for CRISPR 3), followed by 72 °C for 4 min (for CRISPR 1 and 2) or 72 °C for 45 sec (for CRISPR 3) for a total of 40 cycles. Finally, the last extension was carried out at 72 °C for 7 min (McGhee and Sundin, 2012). The amplified PCR products were separated by gel electrophoresis on a 1% (w/v) agarose gel in 0.5 × TAE buffer, stained with ethidium bromide, and analyzed under a gel transilluminator for 3 hours.

Table 1. Primers and oligonucleotide sequences used for the molecular diagnosis and characterization of *E. amylovora* strains obtained from infected pear trees.

| Primer name | Gene region | Primer sequence (5′-3′) | Amplicon size (bp) | Reference |
|---|---|---|---|---|
| A-B primer pairs | pEA29 plasmid | 5’CGGTTTTTAACGCTGGG 3’ | 900-1000 | Lecomte *et al*., 1997; Bereswill *et al*., 1992 |
| | | 5’GGGCAAATACTCGGATT- 3’ | | |
| amsG primer pairs | Ams | 5’GCTTTATGGCACGGATATGG3’ | 117 | Pester *et al*., 2012 |
| | | 5’CCAACGAGATCGAAGGTACG3’ | | |
| CR1-F1_EA / C1-R0_EA primer pairs | CRISPR 1 | 5'CGCCGCCACGCTGCCATTT3' | 300~2200 | McGhee and Sundin, 2012 |
| | | 5'TCCAGCGCCTGTAAAGCGGC3' | | |
| Cr2-F1_EA / C2-R1_EA primer pairs | CRISPR 2 | 5'GCGGCCAACAGATGCGGAAAG3' | 80~2500 | McGhee and Sundin, 2012 |
| | | 5'TGCGGGGAACACTCGACATCTAAT3' | | |
| CR3-F1_EA / CR3-R1_EA primer pairs | CRISPR 3 | TTTTCGCCGGGTAACAGG | 500 | McGhee and Sundin, 2012 |
| | | ATGAGAAGCCCGTGAAGCAAAGTA | | |

The PCR amplicons were separated by electrophoresis using a 1.5% (w/v) agarose gel stained with ethidium bromide in 1×TBE buffer. The lengths of the PCR products were determined using a DNA molecular weight marker (Fermantas 1 kb Plus DNA Ladder SM 1153).

**Evaluation of Disease Index and Severity**

The disease symptoms in pear seedlings were assessed, and the percentage disease index and severity were determined through measurements after the leaf evaluations were completed.

The experiment was conducted using a completely randomized block design with five replications. Each replication represented the average of three shoots from a single seedling (Duzgunes *et al*., 1987). Seven days after the bacterial inoculation, the plants were evaluated on a 0-10 scale for disease index (%) as detailed below:

0 No visible symptoms were observed,
1 The main leaf vein showed slight browning at the cutting point (3-5 mm),
2 Browning of the main leaf vein extended beyond the cutting point (greater than 5 mm),
3 The entire main leaf vein was brown from the cutting point,
5 Both the main and lateral leaf veins exhibited browning and/or necrosis from the cutting point, affecting half of the leaf,
7 The entire leaf turned brown, with necrosis advancing to the petiole,
10 There was necrosis in all leaf veins, along with infection in the shoot

Disease index (%) was evaluated according to the following formula:

$$\textit{Disease Index}\ (\%) = \left[ \frac{\Sigma\ (dln \times sc_1) + \ldots\ldots + (dln \times sc_{10})}{(tln \times sc_{10})} \right] x\ 100$$

(dln: diseased leaf number, sc: symptom class, tln: total leaf number).

The length of visible blighted lesions was compared to the current season's shoot length, with a clear boundary observed between diseased and healthy tissues. The ratio was recorded after all lesions had ceased to grow. The disease severity (DS, %) was determined using the equation below:

$$DS\ (\%) = (a / b) \times 100$$

where a represents the length of the wilted part of the shoot (cm), while b denotes the total length of the shoot (cm) (Fernando and Jones, 1999; Aldwinckle *et al*., 2001).

**Statistical Analyses**

The data obtained from the study were analyzed using MINITAB ver. 14 for variance analysis, and statistical evaluations were conducted with the MSTAT program, applying the Tukey multiple comparison test (Duzgunes *et al*., 1987).

## RESULTS

**Isolation, Biochemical and Physiological Tests**

Between 2021 and 2022, a total of 102 isolates were obtained from 85 plant samples collected from pear trees showing fire blight symptoms in 8 different provinces (Antalya, Konya, Bursa, Kahramanmaraş, Afyonkarahisar, Iğdır, Samsun, Nevşehir) across 6 different regions. Additionally, 78 representative strains were purified. The results of the biochemical and physiological tests performed on these strains are provided in Table 2.

Of the total 102 strains obtained, 78 were Gram-negative, facultative anaerobic, oxidase-negative, catalase-positive, esculin and arginine dihydrolase tests- negative, pectolytic activity-negative and acid production from sucrose-negative, erythritol-negative, sorbitol -positive, mannitol -positive, and inositol-negative, gelatin hydrolysis-positive, levan-producing on NSA (containing 5% sucrose), and non-fluorescent on King's B medium. The remaining 24 strains were identified as the pathogen *Pseudomonas syringae* pv. *syringae* and biocontrol agents by phenotypic tests.

**Pathogenicity, HR and Immature Pear Tests**

All strains induced hypersensitive reactions in tobacco leaves 24-48 hours after inoculation. In the test on raw pear fruits, all pear strains isolated in the study caused different amounts of bacterial exudate. In pathogenicity tests carried out on Santa Maria pear seedlings, initial wilting and eventually typical blight and shepherd's crook symptoms were observed in the shoots, and the strains with the highest disease severity were identified as ARADY5, ARAA2-6 and ARAA1-1 from Kahramanmaraş province (Figure 2, Table 2 and Figure 3).

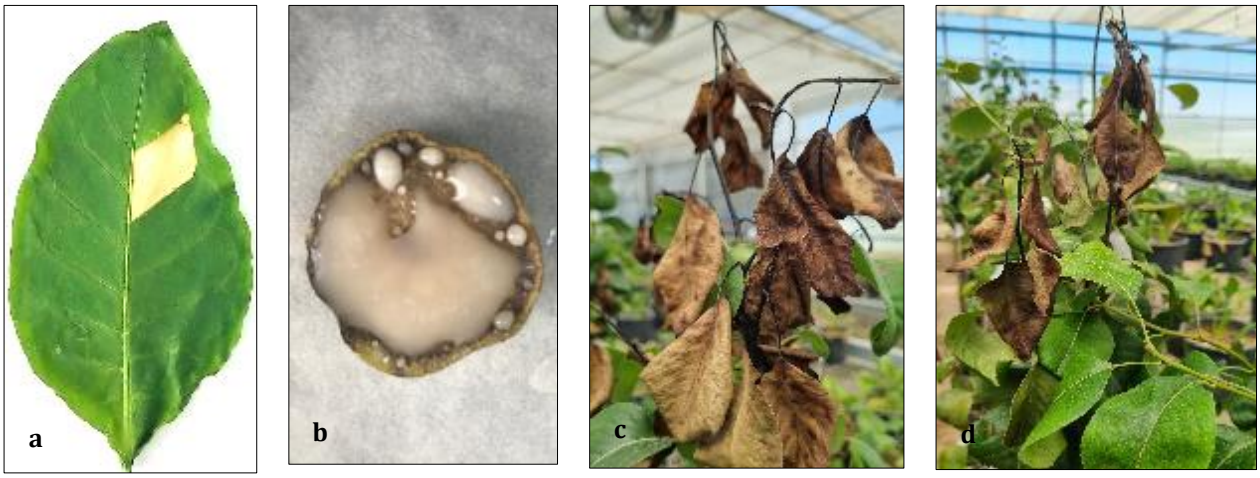


Figure 2. Pathogenicity tests of *Erwinia amylovora* pear strains obtained in the study: (a) hypersensitive reaction on tobacco leaves, (b) ooze formation on immature pears and typical fire blight symptoms in artificial inoculations on Santa Maria variety pear shoots.

In this study, the strains with high virulence (100%) were obtained from the Kahramanmaraş province. Of the 78 strains obtained, 16.66% caused a disease severity of over 80% and were grouped separately in the visual shown in Figure 3. Meanwhile, 83.34% of the strains exhibited lower virulence. Additionally, it was determined that 20 of the 78 isolates were avirulent, and most of these avirulent strains were collected from Konya.

Table 2. Disease severity (%) and disease index (%) caused by pear strains isolated from different provinces of Turkey on shoots of the Santa Maria variety.

| Strains | Provinces | Disease Severity (%) | Disease Index (%) | Strains | Provinces | Disease Severity (%) | Disease Index (%) |
|---|---|---|---|---|---|---|---|
| EA29 (referance strain) | Culture Collection | 100.00 A | 100.00 A | ANTKAR3 | Antalya | 26.99O-S | 19.44NO |
| ARADY5 | Kahramanmaraş | 100.00 A | 100.00 A | ANTKAR15 | Antalya | 26.79N-S | 13.14 RS |
| ARAA2-6 | Konya | 100.00 A | 100.00 A | BDAR2 | Bursa | 26.56N-S | 21.21M |
| ARAA1-1 | Kahramanmaraş | 100.00 A | 100.00 A | ARDKK6 | Konya | 25.21O-T | 11.5 UV |
| ARAA1-3 | Kahramanmaraş | 91.18AB | 100.00 A | MG9AR | Konya | 25.00O-T | 11.7UV |
| ARAA2-5 | Kahramanmaraş | 90.63AB | 100.00 A | MG10AR | Konya | 23.68O-T | 13.8 RS |
| ARADY2 | Kahramanmaraş | 86.79ABC | 100.00 A | EASV5 | Konya | 21.21P-U | 21M |
| 27EA | Konya | 86.67ABC | 100.00 A | KMANAR4 | Konya | 20.72P-V | 20L |
| ARAA1-5 | Konya | 86.36ABC | 100.00 A | NEVAR5 | Nevşehir | 20.63p-v | 34.64 G |
| EASV4 | Konya | 83.52ABC | 100.00 A | ARAA1-6 | Konya | 20.00p-v | 10.9 X |
| ARADY6 | Konya | 83.33ABC | 100.00 A | ARADYSV7 | Kahramanmaraş | 19.64Q-V | 20 L |
| ARAA2-10 | Konya | 82.35ABC | 100.00 A | EA38 | Konya | 19.44Q-V | 19O |
| ARAA7-1 | Konya | 80.00BCD | 100.00 A | ARAA2-2 | Konya | 18.45Q-V | 20 L |
| ARAA2-9 | Konya | 80.00BCD | 100.00 A | MG2AR | Konya | 13.69R-V | 13.1 RS |
| ARADY4 | Konya | 70.00 CDE | 100.00 A | AFSULAR19 | Afyon | 13.14R-W | 18.4O |
| KMANKAR | Konya | 69.64C-F | 100.00 A | ARAA4-4 | Konya | 11.51S-W | 10.55 X |
| EA30 | Konya | 62.86 D-G | 100.00 A | NEVAR1 | Nevşehir | 7.14T-W | 12 TU |
| ARAA4-3 | Konya | 60.29 E-H | 100.00 A | ARDKK5 | Konya | 3.53U-W | 13.35RS |
| EAS26 | Konya | 58.61 E-I | 49.00B | EASV6 | Konya | 2.50VW | 12.55 ST |
| ARAA2-4 | Konya | 58.36E-I | 45.00C | ANTKAR5 | Antalya | 0.00W | 9.6X |
| ARAA1-2 | Konya | 54.41E-J | 45.85 D | IĞAR-1 | Iğdır | 0.00W | 15P |
| ANTKAR6 | Antalya | 53.98E-K | 38F | EASV10 | Konya | 0.00W | 0 Y |
| ANTKAR11 | Antalya | 53.98E-K | 38 F | EASV27 | Konya | 0.00W | 0 Y |
| AFSULAR6 | Afyon | 53.98E-K | 35 G | EA9 | Konya | 0.00W | 10.3 X |
| KMNAR | Konya | 51.58F-K | 35 G | EA24 | Konya | 0.00W | 9.46 X |
| ARAA7-5 | Konya | 51.31F-K | 40E | BDAR1 | Bursa | 0.00W | 0Y |
| ARADY7B | Kahramanmaraş | 50.88G-K | 35G | ARAM4K8-12 | Konya | 0.00W | 0 Y |
| ARADY7 | Kahramanmaraş | 50.88G-K | 35 G | ARAM 4K-14 | Konya | 0.00W | 0 Y |
| ARAA4-1 | Konya | 50.00G-L | 35 G | ARADY3 | Kahramanmaraş | 0.00W | 15 P |
| ARAA7-2 | Konya | 48.84G-M | 38 F | ARADB2 | Bursa | 0.00W | 5 Y |
| ARAA1-8 | Konya | 43.75H-N | 25K | ARADB1A | Bursa | 0.00W | 0 Y |
| AFSULAR7 | Afyon | 41.31I-O | 28V | ARADB1-B | Bursa | 0.00W | 0 Y |
| ARAM3K-2 | Konya | 38.24J-P | 28 IJ | ARAA7-4 | Konya | 0.00W | 4.6 Y |
| ARAA 2-3 | Konya | 35.71K-Q | 27.58O-S | ARAA5.3 | Konya | 0.00W | 0 Y |
| 18EA | Konya | 35.71K-Q | 32H | ARAA5-2 | Konya | 0.00W | 0 Y |
| ARAA7-3 | Konya | 32.26L-Q | 30 IJ | ARAA2-8 | Konya | 0.00W | 0 Y |
| ARAA4-2 | Konya | 30.95M-R | 30.5 IJ | ARAA2-7 | Konya | 0.00W | 0 Y |
| 24EA | Samsun | 30.59M-R | 30.4 IJ | ARAA2-1 | Konya | 0.00W | 0 Y |
| ARAM4K-13 | Konya | 30.36N-R | 30.8 IJ | | | | |
| MG11AR | Konya | 28.42N-S | 28 IJ | | | | |

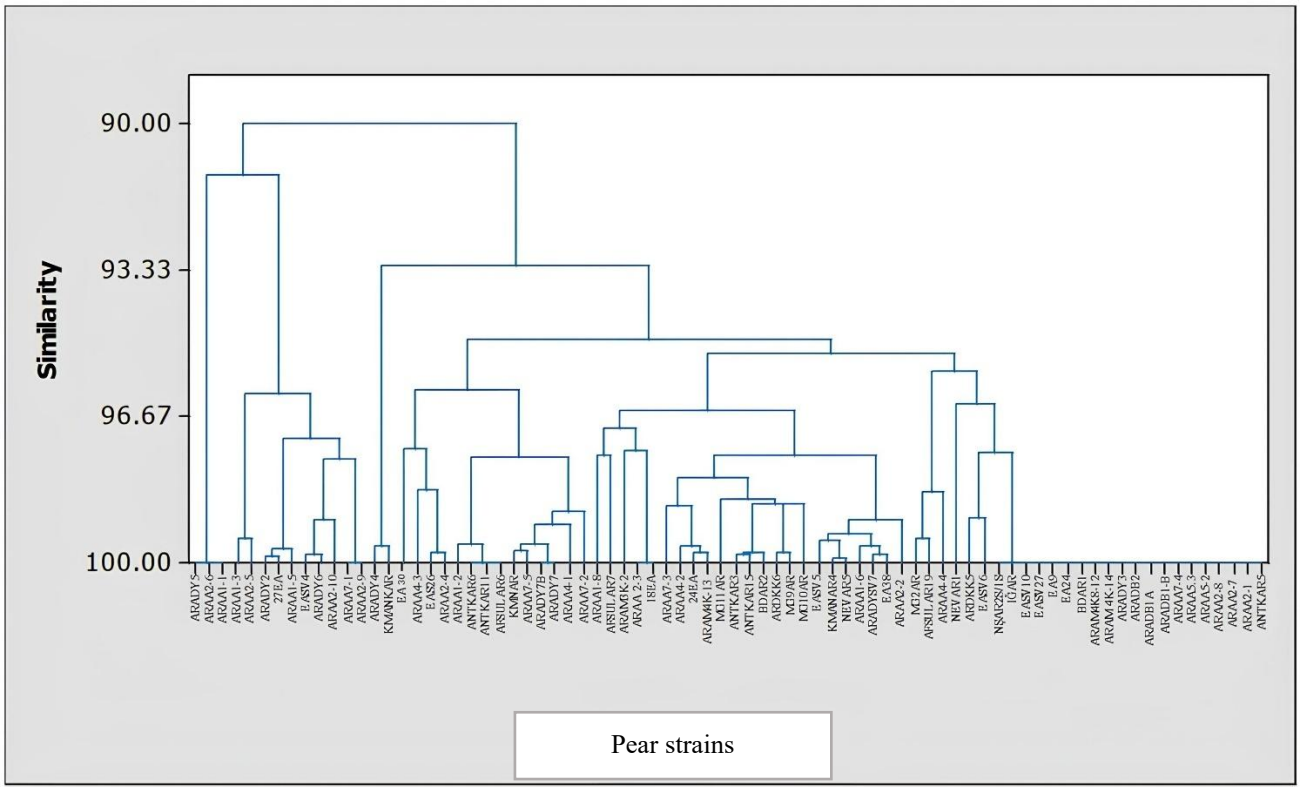


Figure 3. Grouping showing the disease severity (%) of pear strains isolated from different provinces of Turkey on shoots of the Santa Maria variety.

**Molecular Identification and Genetic Diversity of *E. amylovora* Strains**

According to the biochemical, physiological and pathogenicity test results, 78 pear strains identified as *E. amylovora* were characterized with amplicons of 900 bp with A/B primers and 117 bp with amsG primers (Figure 4). All 78 obtained strains produced PCR amplicons of the expected size with both A/B and amsG primers and no genetic differences were detected. The results indicates that these primers, which are generally used for diagnostic purposes, are not sufficient to reveal genetic variability.

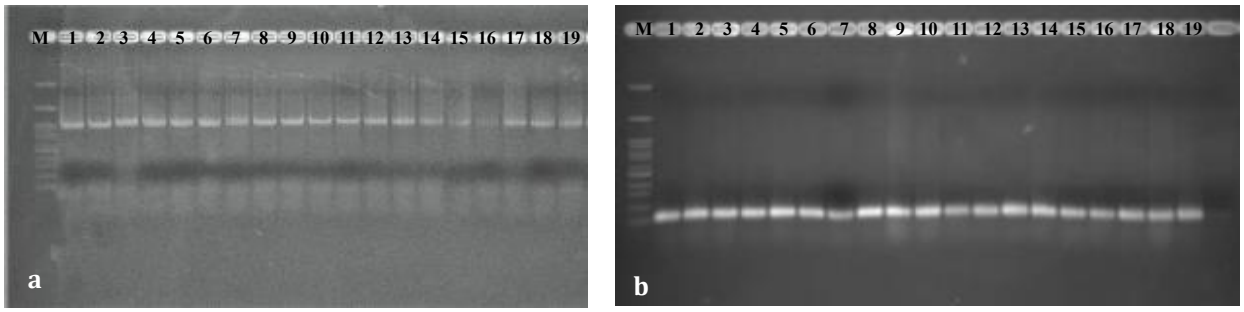


Figure 4. PCR amplification fragments of some *Erwinia amylovora* pear strains isolated from different provinces of Turkey with A/B and amsG primers. Gel image obtained with (a) A/B primers and (b) amsG primers (M: marker (1000 bp, 1: ARADY5, 2: ARAA2-6, 3: ARAA1-1, 4: ARAA1-3, 5: ARAA2-5, 6: ARADY2, 7: ARAA2-2, 8: MG2AR, 9: AFSULAR19, 10: ARAA4-4, 11: ANTKAR3, 12:ANTKAR15, 13: BDAR2, 14: ARDKK6, 15: AFSULAR7, 16: ARAM3K-2, 17: ARAA 2-3, 18: 18EA, 19: EA29 (reference strain).

**CRISPR Arrays**

In the scope of the study area, *E. amylovora* strains with varying virulence levels were selected from eight provinces to represent each region and were utilized in CRISPR arrays. The eight selected strains collectively displayed identical band patterns for CRISPR 1, ranging approximately from 300 to 2200 bp (Figure 5a). For the CRISPR 2 spacer, band patterns varied from approximately 80 to 1600–2500 bp (Figure 5b), although the Nevşehir province strain (NEVAR5) produced a distinct amplicon from the others. For the CRISPR 3 spacer, all pear strains generated a single band of 500 bp (Figure 5c). No genetic differences were identified between the strains for CRISPR 1 and CRISPR 3 spacers due to the conserved regions present in these genes. The results suggest that the CRISPR 2 spacer is, to a certain extent, instrumental in elucidating the genetic diversity of *E. amylovora* pear strains. Nevertheless, in this study, differences were observed with CRISPR 2 primers in only a single strain.

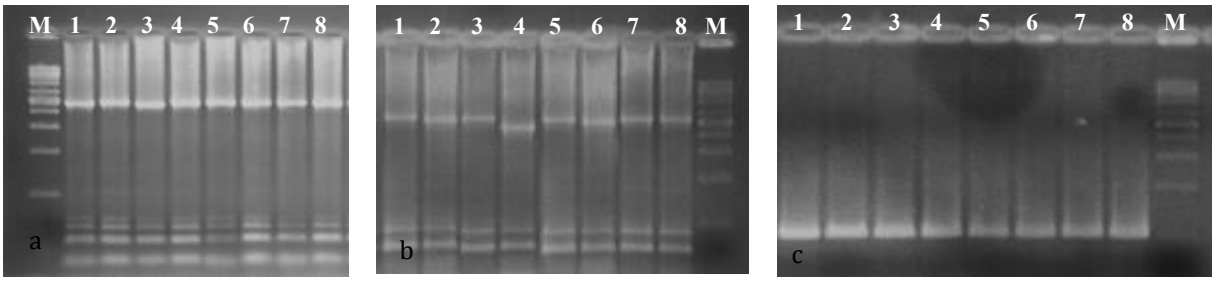


Figure5. CRISPR PCR amplification fragments of some *Erwinia amylovora* pear strains isolated from different provinces of Turkey with CRISPR 1, 2 and 3 primers; Gel image obtained with (a) CRISPR 1 primers, (b) CRISPR 2 primers and (c) CRISPR 3 primers (M: VC 1 kb ladder, 1: ARADY5, 2: ARAA2-6, 3: AFSULAR7, 4: NEVAR5, 5: BDAR2, 6: 24EA, 7: IĞAR-1, 8: ANTKAR15).

**Interaction Between Genetic Variability and Disease Severity of Pear Strains**

In the CRISPR analyses of the study, pear strains selected to represent eight different provinces exhibited virulence levels as follows: ARADY5 (Kahramanmaraş) 100%, ARAA2-6 (Konya) 100%, AFSULAR (Afyonkarahisar) 41.31%, BDAR2 (Bursa) 26.56%, 24EA (Samsun) 30.59%, IĞAR-1 (Iğdır) 0.0%, NEVAR5 (Nevşehir) 20.63%, and ANTKAR15 (Antalya) 26.79%. In addition, it was determined that all strains contained the pEA29 plasmid DNA and possessed the gene responsible for producing amylovoran. The CRISPR 2 spacer analysis, which yielded the best results in determining genetic diversity, showed genetic variation only in the NEVAR5 strain when compared to the others. Despite the substantial differences in disease severity percentages among the strains, no significant correlation was found between virulence levels and the genetic region identified by the CRISPR 2 spacer.

**DISCUSSION**

Significant economic losses occur worldwide due to fire blight disease. It is known that the disease agent has caused major epidemics in the past, leading to millions of dollars in losses. The agent infects 140 plant species belonging to 37 genera within the Rosaceae family, which includes soft-skinned fruit types such as pear, apple, quince, and loquat (Van der Zwet and Keil, 1979; Vanneste, 2000; Gusberti *et al*., 2015).

In Turkey, fire blight disease was first detected in 1998 in pear trees in the Sultandağı district of Afyonkarahisar. Before the 1990s, the disease was found to have spread in many pear orchards across the country, causing serious damage to the gardens (Momol and Yegen, 1993). Research studies have been conducted to identify areas affected by the disease and assess its distribution, resulting in the determination that the disease is present in varying degrees in pome fruit trees (Tokgönül and Çınar, 1991). In contrast, outside of the Marmara region, pear orchards across the country faced the risk of extinction due to the disease in the 2000s. The pathogen is one of the ten most important bacteria that are still prevalent in Turkey and neighboring countries (EPPO, 2023). EPPO has classified *E. amylovora* as a quarantine pathogen and included it in the A2 quarantine list (EPPO, 2023).

It is more effective in pears and quinces, but in recent years it has been observed that it has caused significant losses in apples in Turkey. In addition, studies have identified the disease in blackberries, rosehips, ornamental apples, firethorns, wild pears, Sorbus, Cotoneaster, and Spirea plants (Bastas and Sahin, 2012a; Bastas and Sahin, 2012b; Bastas *et al*., 2013; Bastas and Ozturk, 2013). The severity and economic impact of these outbreaks vary depending on the virulence and environmental factors of *E. amylovora*, and is a result of its ability to survive endophytically or epiphytically (Thomson, 2000).

In the study, plant samples exhibiting typical fire blight symptoms such as wilting, cankers, shepherd's crook, and bacterial ooze were collected from a total of 85 pear orchards across 8 provinces in 6 different regions of Turkey. According to the biochemical, physiological, and pathogenicity tests, it was determined that 78 of the total 102 strains obtained as *E. amylovora*. Morphological, physiological, biochemical, and pathogenicity tests revealed that the strains were largely homogeneous, and the findings were consistent with the characteristic features reported by Shaad (2001).Various molecular analyses were conducted to differentiate *E. amylovora* strains, and it was generally determined that most of the strains from the Amygdaloideae family displayed homogeneity (McManus and Jones, 1995). Similar results were reported by Popović *et al*. (2020), who noted no differences in the rep-PCR patterns among apple, pear, and quince strains of *E. amylovora*. Bastas (2011) conducted studies on phenotypic and genotypic characterization of *E. amylovora* strains isolated from different hosts, as well as on determining pathogenicity reactions on host and non-host plants and examining the pathogen's pathogenicity gene region. Due to the pathogen's spread via various factors, genetically similar E. amylovora strains from countries with different ecological conditions can rapidly adapt to new environments while retaining their pathogenicity to the same extent as in their original habitats (Brennan *et al*., 2002). The findings of the study suggest that *E. amylovora* is influenced by dynamic ecological and climatic factors across diverse hosts, geographical regions, and countries. This variability may have fostered genetic heterogeneity among the strains examined, indicating that differences in virulence could potentially emerge in strains harboring distinct plasmid DNAs.

The A/B primers used in this study are specific to the pathogen's pEA29 plasmid, which is significant for virulence and plays a role in the synthesis of extracellular polysaccharides (Chiou and Jones, 1993; McGhee and Sundin, 2007). Additionally, it contains the strA and strB genes, which provide antibiotic resistance. In the PCR test conducted with A/B primers, a band between 900–1000 bp was expected, and all bands obtained in the study were 900 bp in size.

The AmsG primers are derived from a gene region involved in amylovoran production, which is 16 kb in length and includes twelve ams genes from AmsA to AmsL (Koczan *et al*., 2009; Wang *et al*., 2009; Langlotz *et al*., 2011). In Turkey, Unlu *et al*. (2021) used specific primers synthesized from the Ams gene region for PCR diagnosis, and in our study, the same primers were successfully used to identify the pathogen. It was also determined that strains unable to produce amylovoran do not cause disease in plants (Wang *et al*., 2012). The virulence of the Ea strains obtained in the study is generally high, with most of them being sourced from Konya province. The strains with the highest virulence in Turkey, namely ARADY5, ARAA2-6, and ARAA1-1, were obtained from Kahramanmaraş province. Genetic analyses and evaluations conducted in the study indicate that the screening of the genes investigated and the subsequent analyses did not provide sufficient information to accurately determine the differences in virulence levels between the strains. It is essential to examine the whole genome sequences of the strains in future studies to obtain findings that could guide subsequent research. CRISPR gene regions regulate the defense systems of bacteria against foreign genetic material, and their effects on virulence genes can also be significant. In some cases, CRISPR can weaken a bacterium's pathogenic potential, while in other cases, by preserving or regulating virulence genes, it can enhance the bacterium's ability to cause infection. Therefore, the relationship between the CRISPR gene region and virulence is highly complex and multifaceted, affecting both defense and pathogenicity.

It has been proven that CRISPR plays an important role in evolutionary and epidemiological studies of bacterial species, as well as in strain identification (Rezzonico *et al*., 2011). Similarly, McGhee and Sundin (2012) differentiated *E. amylovora* strains based on CRISPR amplification sequences and reported that these sequences were successful in selecting genotypes that support three linked sequences in *E. amylovora*. Öztürk

and Kayaarslan (2024) found that, as a result of CRISPR analysis, *E. amylovora* has a more diverse and complex structure compared to previous studies. While similar CRISPR band patterns were observed in all isolates and reference cultures, it was determined that CRISPR 1 and CRISPR 3 sequences were highly conserved and showed the same PCR patterns across all strains. However, genetic variation was found in the CRISPR 2 locus. In our study, most of the strains had a similar CRISPR band pattern. However, the strain obtained from Nevşehir province (NEVAR5) was found to have a different pattern in the CRISPR 2 locus compared to the other strains (Figure 5). This indicates the presence of genetic differences in *E. amylovora* strains from Turkish pears. At the same time, the genetic difference in the NEVAR5 isolate is thought to be due to regional factors, as well as potentially being related to its low virulence. It also suggests that the CRISPR 1 and CRISPR 3 regions show less variability and, therefore, may be more limited in detecting genetic variations related to pathogenicity. Future studies could focus on the CRISPR 2 gene region, as well as conduct a more in-depth analysis of the CRISPR 1 and CRISPR 3 regions to identify potential genetic variations in these areas. Additionally, the use of other genetic analysis methods (such as whole-genome sequencing, SNP analysis, etc.) can contribute to the detection of finer variations and genetic factors associated with pathogenicity in these regions. The relationship between CRISPR regions and virulence levels is thought to involve a highly complex and multifaceted mechanism in terms of both defense and pathogenicity. It was determined that the CRISPR 1, 2, and 3 primers used in our experiments were not sufficient to directly detect the virulence relationship of pear strains. Therefore, further analyses are needed with pear strains obtained from different ecological regions and countries worldwide, as well as with different CRISPR primers.

A full understanding of the origin of the disease and its rapid spread within the country will be possible by expanding the genetic analysis to a larger number of strain covering all fruit growing regions of Turkey and the places of origin of imported plants.

According to the results obtained, it is of great importance to consider the virulence levels in the production areas of the regions where the *E. amylovora* pathogen is detected, and appropriate control measures should be taken according to the prevalence of the disease. Detecting this highly destructive pathogen and understanding its virulence and genetic characteristics are crucial for the epidemiology of the disease.

## CONCLUSION

In this study, the relationships between disease severity and genetic variations of 78 pear strains isolated from 102 samples collected across eight provinces in six different regions of Turkey were examined on the fire blight-susceptible Santa Maria pear variety. While no genetic differences were detected among the Turkish pear strains analyzed in the experiments with specific primers designed for the pEA29 plasmid and the amylovoran-producing gene, differences were identified among strains using the CRISPR 2 primer pair. Genetic analysis involving a larger number of strains, encompassing all fruit-growing regions of Turkey and the origins of imported plants, will be necessary to understand the rapid spread of the disease within the country. Based on the results, it is crucial to consider the virulence levels in production areas where the *E. amylovora* pathogen has been detected and to implement appropriate control measures according to the prevalence of the disease. Detecting this highly destructive pathogen and understanding its virulence and genetic characteristics are vital for the epidemiology of the disease. It was found that the CRISPR primers used in our experiments were insufficient for directly detecting the virulence relationship of pear strains. Therefore, further analysis is needed with pear strains obtained from different ecological regions and countries worldwide, using different CRISPR primers.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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