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*Research Article*

# PHYLOGENETIC STUDY OF *RALSTONIA SOLANACEARUM* IN GEORGIA

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**ABSTRACT**: *Ralstonia solanacearum* is the causative agent of one of the most devastating plant diseases, known as bacterial wilt or brown rot. In the conducted study, it was observed that the majority of the isolates, specifically 19 out of 20, were classified within Phylotype II, Race 3, and Biovar II. This classification aligns with the global distribution patterns of *R. solanacearum*, suggesting a widespread adaptation of this phylogenetic group. The regions of Akhaltsikhe, Khulo, Kobuleti, and Akhalqalaqi were identified as the primary sources of these isolates, highlighting the significant prevalence of the pathogen in these areas. However, a singular isolate (KT47) was identified as Phylotype I, Race 1/Biovar III, marking a distinct genetic lineage and suggesting the presence of diverse evolutionary pathways within Georgia. The phylogenetic analysis revealed that the Georgian isolates of *R. solanacearum* exhibit a considerable degree of genetic diversity. The clustering of the majority of isolates into a single phylogenetic group was noted, with a close relationship to strains from Indonesia indicating a common lineage. However, the distinct classification of one isolate into Phylotype I suggests the introduction of diverse strains into the Georgian agricultural ecosystem, possibly through international trade or other means of transmission.

**Keywords:** *Phytopathogenic bacteria, Ralstonia solanacearum, Plant disease, Phylotype diversit*

## INTRODUCTION

*Ralstonia solanacearum*, a soil-borne bacterium, is the causative agent of one of the most devastating plant diseases known as bacterial wilt or brown rot [1,2]. This pathogen affects a wide range of host plants, with particularly severe impacts on economically important crops such as potatoes and tomatoes. Globally, *R. solanacearum* has been recognized for its ability to cause significant yield losses, leading to considerable economic implications for the agricultural sector [3,4]. Disease management efforts are complicated by the resilience of bacterium, facilitated by its wide host range and ability to survive in diverse environmental conditions. In potato and tomato crops, the disease manifests as wilting, stunted growth, and eventual plant death, resulting in substantial reductions in both yield and quality [5, 6].

*Ralstonia solanacearum* is notable for its broad host range, infecting over 200 plant species across more than 50 botanical families, and for its genetic complexity, which complicates efforts to control the pathogen [7, 8]. Traditionally, *R. solanacearum* has been divided into five races based on host range and six biovars based on biochemical properties [9, 10]. Recent advances in molecular techniques have further refined this classification, leading to the identification of phylotypes and sequevars that reflect the genetic diversity and evolutionary history of the pathogen [11, 12]. The concept of the *Ralstonia solanacearum* species complex

(RSSC) encompasses this diversity, highlighting the need for targeted management strategies that consider the specific characteristics of different strains [13,14].

The genetic diversity of *R. solanacearum* has been extensively studied using various molecular techniques. For instance, 16S rRNA gene sequencing has been a valuable tool for identifying and characterizing different strains of the pathogen [15, 16]. Additionally, analysis of the endoglucanase (egl) and hypersensitive response and pathogenicity (hrpB) genes has provided further insights into the phylogenetic relationships and evolutionary history of *R. solanacearum*. These genetic markers have been used to classify the pathogen into four phylotypes, which correspond to distinct geographical distributions and host ranges. Phylotype I, for example, is predominantly found in Asia and includes biovars 3, 4, and 5, which are responsible for significant crop losses in this region [17, 18].

In Georgia, agriculture plays a pivotal role in the economy, with potatoes and tomatoes being among the key crops cultivated. Potatoes are one of the most significant crops in Georgia, with an annual production of around 300,000 tons [19, 20]. The potato industry contributes approximately 3.5% to the country's agricultural output and employs a substantial portion of the rural population. Tomatoes are another economically vital crop, with an annual production exceeding 180,000 tons. The tomato industry accounts for nearly 2% of Georgia's agricultural output and plays a crucial role in the livelihoods of small-scale farmers. Together, potato and tomato cultivation contribute significantly to Georgia's agricultural sector, which accounts for approximately 8% of the country's gross domestic product (GDP) [21]. However, the presence of *Ralstonia solanacearum* poses a severe threat to the productivity and sustainability of these crops, potentially leading to substantial yield losses and economic implications for farmers [22]. Despite its importance, comprehensive studies on the distribution and genetic diversity of *R. solanacearum* in Georgia are limited.

There are a few previous studies that have documented the presence and impact of bacterial wilt caused by *Ralstonia solanacearum* in Georgia. Mepharishvili et al. (2012) [23] reported the first confirmed case of bacterial wilt of tomato caused by *R. solanacearum* in Georgia. The study identified the presence of the pathogen in tomato fields in the Samegrelo region, a major tomato-growing area in the country. Muradashvili et al. (2014) [24] reported the first occurrence of potato brown rot caused by *R. solanacearum* in Georgia. The study found the pathogen infecting potato crops in the Akhaltsikhe region, known for its potato cultivation. The study revealed the presence of diverse phylotypes, indicating the potential for the emergence of new virulent strains and the need for comprehensive research. These previous studies and reports confirm the presence of *R. solanacearum* in Georgia and highlight the significant impact of bacterial wilt on economically important crops like potatoes and tomatoes. They emphasize the need for further research to understand the distribution, genetic diversity, and epidemiology of the pathogen, as well as the development of effective management strategies tailored to local conditions.

Given the significant impact of *Ralstonia solanacearum* on agriculture, comprehensive studies that integrate genetic, ecological, and management perspectives are urgently needed. This study aims to address this knowledge gap by systematically investigating the distribution and phylogenetic diversity of *R. solanacearum* across Georgia. By achieving this, valuable insights into the epidemiology of bacterial wilt in Georgia will be provided, contributing to the development of targeted control measures and supporting the sustainable cultivation of potatoes and tomatoes. Specifically, the objectives of this study are to map the distribution of *R. solanacearum* isolates across Georgia, assess the genetic diversity of these isolates using molecular markers, and identify potential sources of introduction and dissemination of the pathogen. By addressing these knowledge gaps, the study aims to contribute to the development of targeted disease management strategies that support the sustainable cultivation of potatoes and tomatoes in Georgia. The findings will inform the design of integrated

management strategies that combine cultural, chemical, and biological approaches to control bacterial wilt and enhance the resilience of agricultural systems in Georgia.

## MATERIALS AND METHODS

### *Sampling Strategy*

A comprehensive sampling strategy was employed to investigate the distribution and phylogenetic diversity of *Ralstonia solanacearum* across Georgia's primary agricultural regions. Targeting susceptible host plants such as potatoes, tomatoes, and peppers, the study focused on areas with a known history of bacterial wilt: Akhaltsikhe, Khulo, Kobuleti, and Akhalqalaqi. These regions were selected based on their historical incidence of the disease and the diversity of their climatic conditions, which could influence the pathogen's spread and virulence. Sampling was conducted over four years (2014-2018) during peak harvest periods to ensure a comprehensive collection of isolates (Table 1). This approach allowed for the gathering of a wide array of samples, reflecting the pathogen's current distribution and potential evolutionary trends. Adhering to the protocols outlined in EU Directive 2006/63/CE [25], the strategy aimed to provide a robust dataset for analyzing the pathogen's presence, contributing to the development of targeted control measures and supporting sustainable agricultural practices in Georgia.

### *Isolation and Identification of Ralstonia solanacearum*

The isolation and identification of *Ralstonia solanacearum* were conducted following international standards [26]. Infected plant material was submerged in sterile water for 5 minutes to facilitate the isolation of bacteria. Visible bacterial streams from xylem vessels indicated the presence of the pathogen. A loopful of this bacterial suspension was then cultured on Kelman's tetrazolium chloride medium (TZC) and semi-selective agar medium (SMSA) for 48 hours at 28°C, following the methodology established by Elphinstone [27].

### *PCR Amplification and DNA Sequencing*

For molecular characterization, species-specific Real-Time PCR was utilized to identify the target pathogen, employing TaqMan® chemistry with universal primers OLI1 and Y2, targeting a region that differentiates Ralstonia from other bacteria [28, 29]. The standard strain *R. solanacearum* #325 from the NCPPB collection served as a positive control. Amplification products were visualized on a 1.5% agarose gel stained with ethidium bromide [30].

PCR amplification of a 750-bp region of the egl gene was performed by using the primer pair Endo F/Endo-R. The reaction mixture (total volume, 50µl) contained PCR buffer (supplied by the manufacturer), 1.5 mM MgCl2, 200 µM of each dNTP, 25 pmol of each primer, 2 µl of a bacterial suspension as the template, and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR was performed using a PTC200 thermo cycler, using the following protocol: (i) initial denaturation at 96°C for 9 min; (ii) 30 cycles, with 1 cycle consisting of 95°C for 1 min, 70°C for 1 min, and 72°C for 2 min; and (iii) a final extension step of 72°C for 10 min. Reaction were carried out by the method of Prior and Fagan (2005), [30].

The endoglucanase virulence sequence data were analyzed by the MEGA6 software environment [31]. Cluster analysis was used to build the phylogenetic tree (UPGMA). The data were statistically processed using the maximum likelihood FastDNAML program [32]. 48 reference strains, which are described and deposited into the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast) were included in the analysis along with 20 new Georgian nucleotide sequences (Table 1).

### *Confirmation Tests and Pathogenicity Assessment*

Pathogenicity tests were conducted to confirm the virulence of identified isolates. An inoculant of $10^6$ cells per ml was prepared from the test culture and a positive control strain. Ten tomato plants were inoculated at the third true leaf stage or older and incubated for up to two weeks at 22°C- 28°C under high relative humidity with daily watering. Observed symptoms included wilting, epinasty, chlorosis, and stunting.

The development of disease on host plants was recorded using disease quality scores based on the scale provided by Winstead and Kelman (1952), [33]. The disease index (DI) was subsequently calculated using the Eqn. 1

$$\mathrm{DI} = \left[\frac{\sum(n_i \times v_i)}{V \times N}\right] \times 100$$

**Eqn.1**

where:

- $n_i$ represents the number of plants at each specific disease rating.
- $v_i$ is the value assigned to each disease rating.
- $V$ is the highest value on the disease rating scale.
- $N$ denotes the total number of plants evaluated.

Statistical analysis of the experimental data was performed using the computer program GraphPad Prism 6. A two-factor ANOVA and a multiple-test method were employed. The results obtained indicated that the reliability coefficient varied from P = 0.1 to P = 0.9, with degrees of freedom (df) equal to 4.

Symptomatic plants were further examined for *R. solanacearum* carriage by removing a section of tissue from the stem 2 cm above the inoculation point, which was then comminuted, suspended in sterile distilled water, plated, and incubated to check for typical colonies of *R. solanacearum*, as described by Klement [34].

## RESULTS AND DISCUSSION

In order to ascertain the extent of dissemination of phytopathogenic bacteria *R. solanacearum* and the distribution of its host plants, we have carried out a number of monitoring in various Georgian regions. Three different geographic zones: Kobuleti (10 m), Khulo (923 m), and Akhaltsikhe (1200 m) were selected to reveal the extent of the disease distribution.

Our six-year study findings demonstrated that *R. solanacearum* had already colonized the following regions: Potato brown rot accounted for 63.6% of all plants in Samtckhe-Javakheti; the Colchis lowlands had the highest frequency of occurrence on potatoes (62, 5%) and tomatoes (48, 48%), with an intensity of 48.54%. The disease was spread on a variety of host plants (Fig. 1). The isolates were predominantly sourced from potato plants, with a notable presence in tomato and pepper plants, indicating a broad host range for the pathogen across different climatic conditions within the region.


***Fig. 1***. *Geographical distribution of the main places of surveys of Ralstonia solanacearum in Georgia*

The results of the PCR amplification products on the 1, 5% agarose gel showed that the DNA fragment sizes from experimental strains corresponded to the fragment size of the *R. solanacearum*– reference strain (Fig. 2).

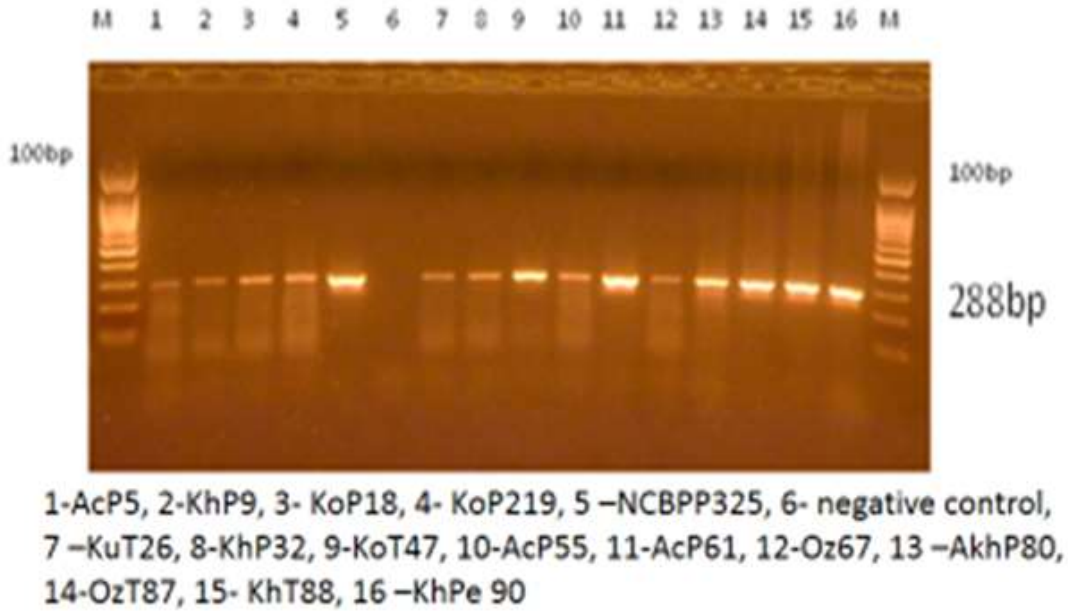


***Fig. 2***. *Electropherogram of DNA amplification product of Georgian isolates of R. solanacearum by OLI1 / Y2 primers.*

In the conducted study, a comprehensive analysis was performed on 20 strains of *Ralstonia solanacearum* isolated from various agricultural zones within Georgia (Table 1).

***Table 1.*** *List of selected Georgian strains of R. solanacearum subjected to Egl sequencing*

| # | BioSample accession: | Georgian isolates | Incident of areal | Host plant | Year | Phylogenetic group | Race/ Biovar |
|---|---|---|---|---|---|---|---|
| 1 | KY922973 | AcP2(M2) | Akhaltsikhe, V.Tsnisi 41°40'13.7"N 43°03'25.8"E | Potato | 2017 | Phylotype I I | R3/BII |
| 2 | KY922974 | AcP5(M5) | Akhaltsikhe, V. Tsnisi 41°40'12.2"N 43°03'15.8"E | Potato | 2017 | Phylotype I I | R3/BII |
| 3 | KY922975 | KhP6(M6) | Khulo, V.Tabakhmela 41°37'59.6"N 42°24'38.3"E | Potato | 2017 | Phylotype I I | R3/BII |
| 4 | KY922976 | KhP9(M9) | Khulo, V.Tabakhmela 41°37'55.6"N 42°24'48.4"E | Potato | 2017 | Phylotype I I | R3/BII |
| 5 | KY922977 | KoP18(M18) | Kobuleti Experimental field 41°48'42.5"N41°47'21.5"E | Potato | 2017 | Phylotype I I | R3/BII |
| 6 | KY922978 | KoPe19(M19) | Kobuleti 41°51'40.3"N 41°49'28.9"E | Pepper | 2017 | Phylotype I I | R3/BII |
| 7 | KY922979 | KhP32(32) | Khulo, V. Didajara 41°39'46.4"N 42°20'48.9"E | Potato | 2017 | Phylotype I I | R3/BII |
| 8 | KY922990 | KhP34(M34) | Khulo, V. Tabakhmela 41°38'01.6"N 42°24'38.6"E | Potato | 2017 | Phylotype I I | R3/BII |
| 9 | KY922989 | AkhP41(M41) | Akhalqalaqi 41°24'19.3"N 43°27'38.7"E | Potato | 2017 | Phylotype I I | R3/BII |
| 10 | KY922991 | AcP55(M55) | Akhaltsikhe, V. Tsnisi 41°40'14.8"N 43°03'18.0"E | Potato | 2017 | Phylotype I I | R3/BII |
| 11 | KY922992 | AcP61(M61) | Akhaltsikhe, V. Tsnisi, 41°40'11.8"N 43°03'18.1"E | Potato | 2017 | Phylotype I I | R3/BII |
| 12 | KY922987 | AcP62(M62) | Akhalcikhe, V.Mskhvirisi, 41°38'24.9"N 42°55'47.5"E | Potato | 2017 | Phylotype I I | R3/BII |
| 13 | KY922980 | AcP57(M57) | Akhaltsikhe, V.Tsnisi 41°40'21.4"N 43°03'52.9"E | Potato | 2017 | Phylotype I I | R3/BII |
| 14 | KY922986 | KoT65(M65) | Kobuleti, V. Khutsubani 41°48'27.2"N 41°50'00.0"E | Tomato | 2017 | Phylotype I I | R3/BII |
| 15 | KY922981 | OzT67(M67) | Ozurgeti, V. Dzimiti 42°00'02.1"N 42°03'36.5"E | Tomato | 2017 | Phylotype I I | R3/BII |
| 16 | KY922982 | AkhP79(M79) | Akhalqalaqi 41°23'33.1"N 43°28'54.7"E | Potato | 2017 | Phylotype I I | R3/BII |
| 17 | KY922985 | AkhP81(M81) | Akhalqalaqi, V. Kotelia 41°31'51.4"N 43°28'30.8"E | Potato | 2017 | Phylotype I I | R3/BII |
| 18 | KY922984 | KhT88(M88) | Khulo, V. Kedlebi 41°39'13.6"N 42°17'16.0"E | Tomato | 2017 | Phylotype I I | R3/BII |
| 19 | KY922983 | KhPe90(M90) | Khulo 41°38'48.2"N 42°18'39.2"E | pepper | 2017 | Phylotype I I | R3/BII |
| 20 | KY922988 | KT47(M47) | Kobuleti 41°51'16.2"N 41°49'45.7"E | Tomato | 2017 | Phylotype I | R1/BIII |

These strains were subjected to *egl* sequencing to elucidate their phylogenetic diversity and distribution patterns (Fig. 3).

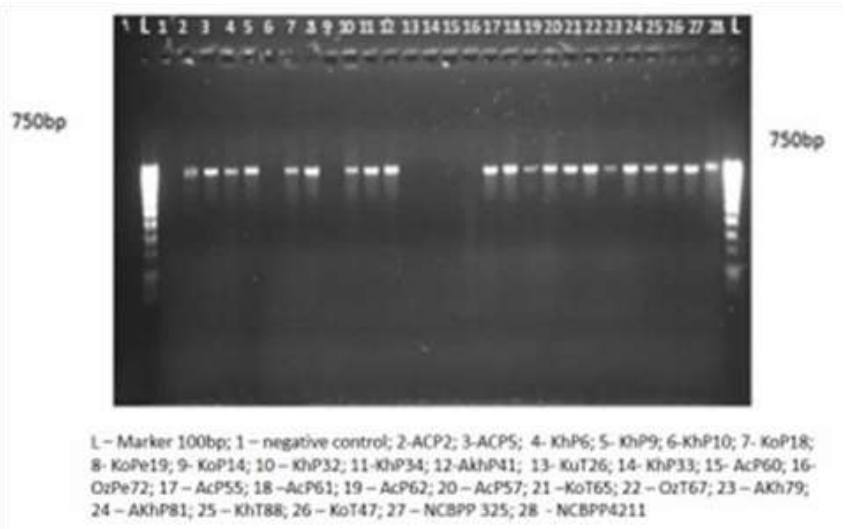


***Fig 3.*** *Electropherogram of the amplified fragment to the endoglucanase gene of R. solanacearum*

It was observed that the majority of the isolates, specifically 19 out of 20, were classified within Phylotype II, Race 3/Biovar II. (Fig. 1). This classification aligns with the global distribution patterns of *R. solanacearum*, suggesting a widespread adaptation of this phylogenetic group. The regions of Akhaltsikhe, Khulo, Kobuleti, and Akhalkalaki were identified as the primary sources of these isolates, highlighting the significant prevalence of the pathogen in these areas. A singular isolate (KT47), however, was identified as Phylotype I, Race 1/Biovar III, marking a distinct genetic lineage and suggesting the presence of diverse evolutionary pathways within Georgia.

The phylogenetic analysis revealed that the Georgian isolates of *R. solanacearum* exhibit a considerable degree of genetic diversity. The clustering of the majority of isolates into a single phylogenetic group was noted, with a close relationship to strains from Indonesia, indicating a common lineage. However, the distinct classification of one isolate into Phylotype I suggests the introduction of diverse strains into the Georgian agricultural ecosystem, possibly through international trade or other means of transmission. When compared with global strains, the Georgian isolates were found to share similarities with those identified in other temperate and tropical regions. This comparison underscores the pathogen's ability to adapt to a wide range of environmental conditions, posing challenges for disease management and control strategies. The identification of a Phylotype I strain among the predominantly Phylotype II isolates in Georgia was particularly noteworthy, as it indicates a broader ecological niche for this group than previously understood.

The findings from this study have been instrumental in highlighting the need for vigilant monitoring and tailored management practices to combat the spread of *R. solanacearum* in Georgia. The genetic diversity observed among the isolates underscores the potential for the emergence of new strains, necessitating ongoing surveillance and research to develop effective control measures.

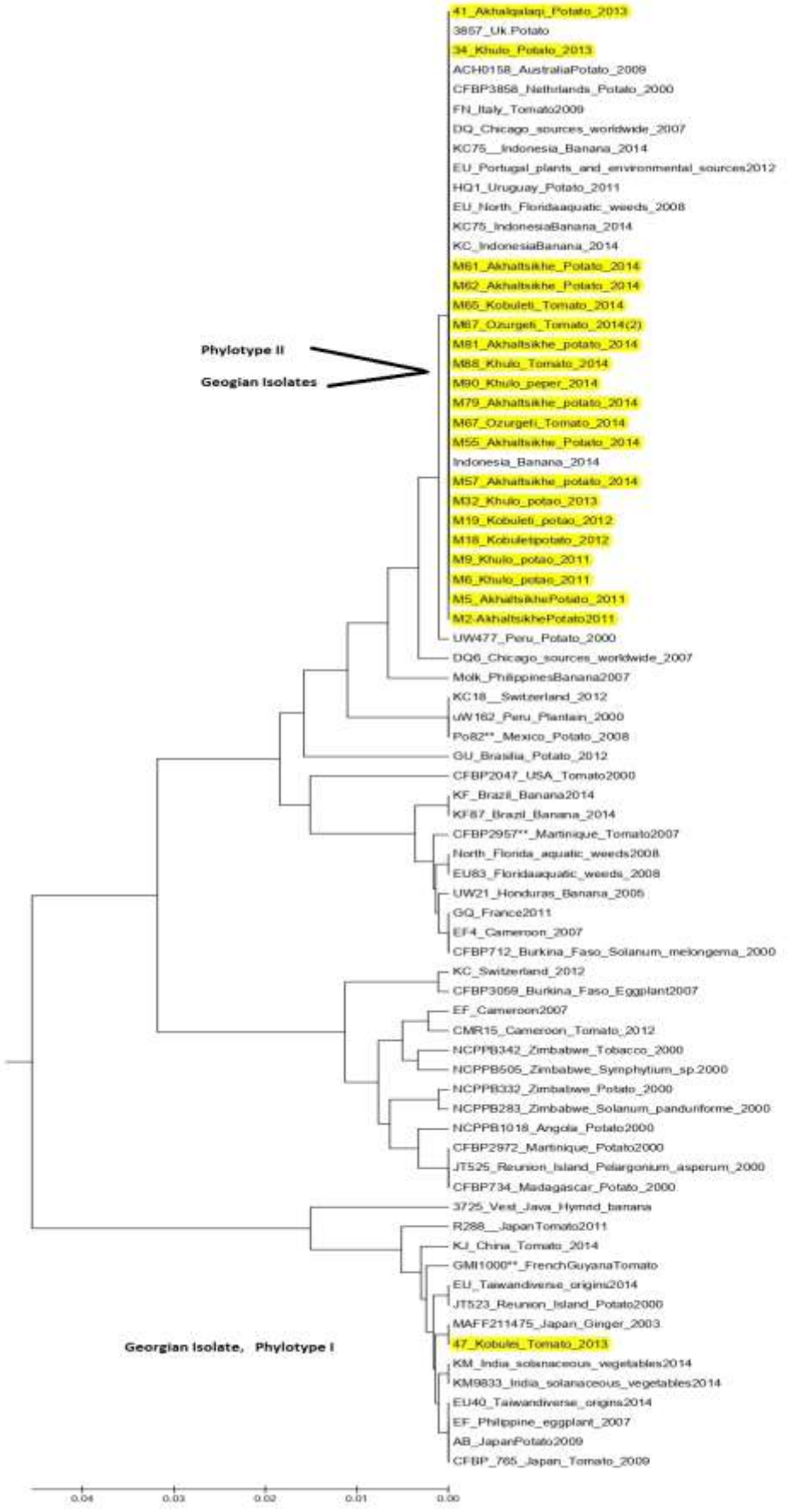


***Fig.4.*** *Phylogenetic tree of R. solanacearum Georgian and reference strains.*

The findings of this study provide comprehensive insights into the distribution and phylogenetic diversity of *Ralstonia solanacearum* in Georgia. The identification of 19 out of 20 isolates as Phylotype II, Race 3/Biovar II aligns with global distribution patterns observed in numerous studies. Prior et al. (2016) documented similar findings, showing a predominant occurrence of Phylotype II, Race 3/Biovar II strains in various regions, particularly temperate zones [35]. This supports the hypothesis that Phylotype II strains have adapted to diverse environmental conditions, facilitating their widespread distribution. Similarly, Fegan and Prior (2005) highlighted the complexity of the *R. solanacearum* species complex, noting the widespread presence of Phylotype II strains and their adaptability to various environmental conditions [30].

The genetic diversity observed in Georgian isolates is noteworthy. The identification of a singular isolate (KT47) as Phylotype I, Race 1/Biovar III suggests the introduction of diverse strains into the Georgian agricultural ecosystem. This finding is consistent with the work of Wicker et al. (2011), who reported similar genetic diversity in *R. solanacearum* populations in Madagascar, indicating multiple introduction events and evolutionary pathways [36].

The close phylogenetic relationship between Georgian isolates and strains from Indonesia suggests a common lineage, potentially facilitated by international trade or other means of transmission. This observation is supported by Jeong et al. (2007), who found that trade and the movement of agricultural products significantly contributed to the dissemination of *R. solanacearum strains* across Asia [37].

The pathogen's adaptability to a wide range of environmental conditions poses significant challenges for disease management. Genin and Denny (2012) emphasized the resilience of *R. solanacearum,* highlighting its ability to thrive in diverse climates and affect a wide range of host plants [38]. This adaptability was also discussed by Peeters et al. (2013), who noted that the pathogen's wide host range complicates management efforts and necessitates the development of comprehensive control strategies [39]

The discovery of a Phylotype I strain among predominantly Phylotype II isolates suggests a broader ecological niche for *R. solanacearum* than previously understood.

The observed genetic diversity and the potential for new strain emergence necessitate ongoing surveillance. Mansfield et al. (2012) discussed the evolutionary potential of *R. solanacearum* and the importance of vigilant monitoring to detect new strains and implement effective control measures [40]. This necessity for continuous surveillance was also emphasized by Kannan et al. (2015), who highlighted the role of integrated disease management strategies in mitigating the impacts of plant pathogenic bacteria [41].

In conclusion, the study advances the understanding of *R. solanacearum* distribution and genetic diversity in Georgia, providing valuable data for comparative studies and global disease management efforts. The findings emphasize the need for international collaboration in research and plant disease management to address the challenges posed by this pathogen effectively.

## CONCLUSION

This comprehensive study on the distribution and phylogenetic diversity of *Ralstonia solanacearum* across Georgia has provided pivotal insights into the epidemiology of one of the most devastating plant pathogens affecting key crops. Through the detailed analysis of 20 isolates collected from various agricultural zones, we have illuminated the pathogen's widespread presenceand its broad host range, encompassing potatoes, tomatoes, and peppers. The majority of these isolates were identified as Phylotype II, Race 3/Biovar II, aligning with the global prevalence of this pathogen, yet the discovery of a singular Phylotype I, Race 1/Biovar III isolate underscores the genetic diversity and potential for adaptation within the *R.*

*solanacearum* population in Georgia.

The findings from this study underscore the critical need for ongoing surveillance, research, and the development of integrated disease management strategies tailored to the specific conditions and challenges faced by Georgian agriculture. The observed genetic diversity among the *R. solanacearum* strains, particularly the presence of a Phylotype I strain, highlights the potential forthe introduction of new strains through global trade and the necessity for stringent biosecurity measures to prevent such introductions. Furthermore, this research contributes to the global body of knowledge on *R. solanacearum*, offering valuable data for comparative studies and the development of broader strategies to combat bacterial wilt. It emphasizes the importance of international collaboration in research and plant disease management to address the challenges posed by this pathogen effectively.

In conclusion, the study not only advances our understanding of *R. solanacearum* distribution and genetic diversity in Georgia but also lays the groundwork for future research directions. These include exploring the environmental and agronomic factors influencing disease prevalence, evaluating the efficacy of control strategies, and ultimately supporting the sustainable cultivation of susceptible crops. By addressing the challenges posed by R. solanacearum, we can safeguard agricultural productivity and food security in Georgia and beyond.

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