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Occurrence of plant pathogenic *Pseudomonas syringae* in the Danube River Basin: abundance and diversity assessment

Marina Anteljević[1], Iva Rosić[1], Olja Medić[1], Stoimir Kolarević[2], Tanja Berić[1], Slaviša Stanković[1] and Ivan Nikolić[1*]

Abstract

Plant pathogenic strains of *Pseudomonas syringae* (*Psy*) spp. have been detected in nonagricultural habitats, including those associated with the water cycle. Their presence in aquatic systems allows dissemination over long distances, especially with irrigation practices. In May 2021, we sampled 15 sites along the Danube River Basin in Serbia to gain insight into *P. syringae* abundance and diversity. We identified 79 *Psy* strains using *Psy*-specific primers, and a partial sequence of the citrate synthase (*cts*) house-keeping gene has served for phylogenetic diversity assessments. Phenotypic diversity determination included characterizing features linked with survival and pathogenic lifestyle. The ice nucleation activity, pectinolytic activity, swimming and swarming assays, and hypersensitive reaction on plants were tested. *Psy* was detected at ten of 15 sites examined at abundance ranging from 1.0×10^2 to 1.2×10^4 CFU/L. We discovered the presence of four phylogroups, with phylogroup 2 being the most abundant, followed by phylogroups 7, 9, and 13. The hypersensitive reaction was induced by 68.63% of the isolates from the collection. A partial sequence comparison of the *cts* gene showed 100% similarity between isolates from cherry plants epidemics in Serbia caused by *Psy* and isolates from the Danube River. Our results suggest that the Danube River, extensively used for irrigation of agricultural fields, harbors diverse strains of *Psy,* which possess various features that could lead to potential disease outbreaks on crops. This study represents the first in-depth analysis of *Psy* abundance and diversity in the Danube River Basin. It sets the ground for future pre-epidemic studies and seasonal monitoring of *Psy* population dynamics.

Keywords *Pseudomonas syringae*, Abundance, Diversity, Phylogeny, Ice nucleation activity, Motility

Background

Pseudomonas syringae (*Psy*) is a well-studied, widespread complex of plant pathogenic bacteria, a causative agent of diseases on many species, including herbaceous and woody plants (Gutiérrez-Barranquero et al. 2019). Symptoms of the diseases caused by this complex of bacteria can vary from mild to severe. Plants can develop localized manifestations, such as leaf spots, blight, specks, and wilting. Some pathogen species can move through vascular tissue and induce systemic disease (Donati et al. 2020). Similar to many other plant pathogens, the evolution of its virulence has been widely investigated from an agro-centric perspective based on interaction

*Correspondence:
Ivan Nikolić
ivan.nikolic@bio.bg.ac.rs
[1] Faculty of Biology, Center for Biological Control and Plant Growth Promotion, University of Belgrade, Studentski Trg 16, Belgrade 11000, Serbia
[2] Institute for Biological Research 'Siniša Stanković', National Institute of Republic of Serbia, Department of Hydroecology and Water Protection, University of Belgrade, Bulevar Despota Stefana 142, Belgrade 11000, Serbia

with its primary hosts (Morris et al. 2009; Scortichini et al. 2012). However, over the past decade, reports have been increasing on the importance of alternative plant hosts and habitats beyond agroecosystems (Morris et al. 2008, 2010). The presence of *P. syringae* was confirmed in habitats correlating to the water cycle, such as rivers, lakes, rainwater, snow, and clouds (Morris et al. 2008). The production of ice-nucleating proteins in some *P. syringae* strains enables them to catalyze ice formation at warmer temperatures than temperatures at which pure water freezes (Maki et al. 1974). It was hypothesized that by catalyzing ice formation in clouds, bacteria provide a deposition to nutrient-rich niches through precipitation (De Araujo et al. 2019). Moreover, ice nucleation activity also threatens plant health, increasing the risk of frost injury (Karimi et al. 2020).

Many of the *P. syringae* hosts are economically important crops, so it is essential to investigate all potential reservoirs of the bacteria. In that context, freshwater habitats are important as they are used for irrigation purposes in agricultural areas and are possible sources of pathogen inoculum. There are many reports about plant pathogenic bacteria in open irrigation systems, such as those from genera *Xanthomonas*, *Pectobacterium*, and *Dickeya* (Faye et al. 2018; Fayette et al. 2018; Hugouvieux-Cotte-Pattat et al. 2019). *P. syringae* was previously detected in freshwater by Morris et al. (2008, 2010), in a freshwater lake in Virginia by Pietsch et al. (2017), also in the study of *Pseudomonas* species diversity along the Danube (Mulet et al. 2020). After cantaloupe blight epidemics caused by *P. syringae* appeared in Southern France in 1993, a post-epidemic study of *P. syringae* in water retention basins was conducted, and the causative agent was isolated from there (Riffaud and Morris 2002). Similarly, for the plant pathogenic fungus *Verticillium dahlia*, irrigation was the primary dispersal means to cause the contamination (Baroudy et al. 2018). Repeated irrigation from the same contaminated water source is an additional danger because the pathogen is reintroduced into the field (Parke et al. 2019). One of the biggest concerns is the emergence of new and highly aggressive populations of plant pathogenic bacteria, considering the enormous genetic diversity in environmental reservoirs. A much broader diversity of plant pathogenic *P. syringae* was obtained from alpine headwaters compared to those isolated from infected plants (Morris et al. 2010; Berge et al. 2014). *P. syringae* environmental isolates, closely related to *P. syringae* pv. *tomato*, had a broader host range but caused almost the same disease symptoms when inoculated in the laboratory under optimal conditions (Monteil et al. 2013). Moreover, *D. dianthicola* isolates from the rivers were more aggressive on potatoes than strains isolated from diseased plants (Laurila et al. 2008). Environmental strains surrounded by various microbial species have higher chances of acquiring new loci by horizontal gene transfer (Dillon et al. 2019). Insights into the abundance of *P. syringae* in aquatic habitats and assessment of its diversity are important to evaluate its suitability for irrigation. Water inhabited with pathogens is a possible infection source and can lead to epidemics with consequential yield losses (Monteil et al. 2016).

The Danube is the second-longest river in Europe that links more countries than any other river in the world. It flows through or along the borders of ten European countries (Dávid and Madudová 2019). It is the longest river in the Black Sea Basin of Serbia, collecting waters from 92% of Serbian territory. The development of a strategy for irrigation and drainage in the Republic of Serbia is in progress, which will inevitably include the Danube-Tisa-Danube canal network from the territory of Vojvodina. Evaluation of irrigation water quality based on chemical parameters was done for groundwater sources of the Danube (Kurilić et al. 2019) and surface water in the Danube-Tisa-Danube hydrosystem area, encompassing 75% of arable land (Zemunac et al. 2021). Both studies confirmed suitability for irrigation purposes but were based only on chemical characteristics.

Regarding microbial contamination which can potentially cause a severe threat to human health, fecal microbial pollution is a major problem in the Danube River Basin and is the focus of many investigations (Kirschner et al. 2017; Frick et al. 2020; Banciu et al. 2021). If one considers it a potential irrigation source, it is also essential to monitor plant pathogens. To our knowledge, studies of *P. syringae* in the Danube have not been reported yet.

Our study aimed to estimate abundance and characterize *Psy* isolates from the water samples collected at the sites situated at the Danube River and its major tributaries in the territory of the Republic of Serbia and to determine their phenotypic and phylogenetic diversity. Isolates were identified by PCR reaction using species-specific primers. Their characterization was achieved through biochemical tests, hypersensitive reaction tests on *Pelargonium* plants, ice nucleation activity assay, motility assays, and *cts* gene-based phylogenetic analysis. The Danube and its tributaries were the aquatic habitats of choice, considering the Danube River Basin's potential and irrigation usage. This study provides a basis for a comprehensive pre-epidemic study of *P. syringae* in freshwaters in the context of irrigation, which should provide information on *Psy* community composition, abundance, and diversity.

Results

Abundance of *P. syringae* in the Danube River

Bacterial populations were evaluated for 15 sites along the Danube River Basin in Serbia (Fig. 1 and Additional file 1: Table S1). The total number of *Pseudomonads* spp. cultured on semi-selective media KBC ranged from 2.6×10^3 to 1.45×10^5 CFU/L (Additional file 1: Table S1). The total number of colonies cultured for each site was counted (D1—89, D2—85, D3—86, D4—145, D5—82, D6—95, D7—101, D8—102, D9—26, D10—91, D11—89, D12—69, T1—59, T2—52, T3—86 colonies). We treated all colonies as putative *Psy* isolates and performed PCR identification on all colonies grown on the filter. We performed a colony-PCR reaction using *Psy*-specific primers to detect *Psy* and reveal its abundance in the Danube. From the DNA of the isolates identified as *P. syringae,* 144-bp DNA fragments were amplified when *Psy*-specific primers were used (Guilbaud et al. 2016). The specific band was amplified for 79 isolates belonging to the *P. syringae* species complex. Of 15 sampling sites, *Psy* was detected in ten, while none of the colonies tested were identified as *Psy* in the rest five sites (Fig. 1). The abundance of *P. syringae* varied from 1.0×10^2 to 1.0×10^4 CFU/L (Fig. 1 and Additional file 1: Table S1). The highest abundance was present at locality Tisa Titel (T1) with 1.2×10^4 CFU/L, and the lowest abundance was 1.0×10^2 CFU/L at localities Donji Milanovac (D8) and Tekija (D9).

Phylogenetic analysis

Further confirmation of the isolate identities was obtained by amplification and sequencing of the partial house-keeping citrate synthase (*cts*) gene. In 51 isolates, the *cts* gene had a significant percentage of identity

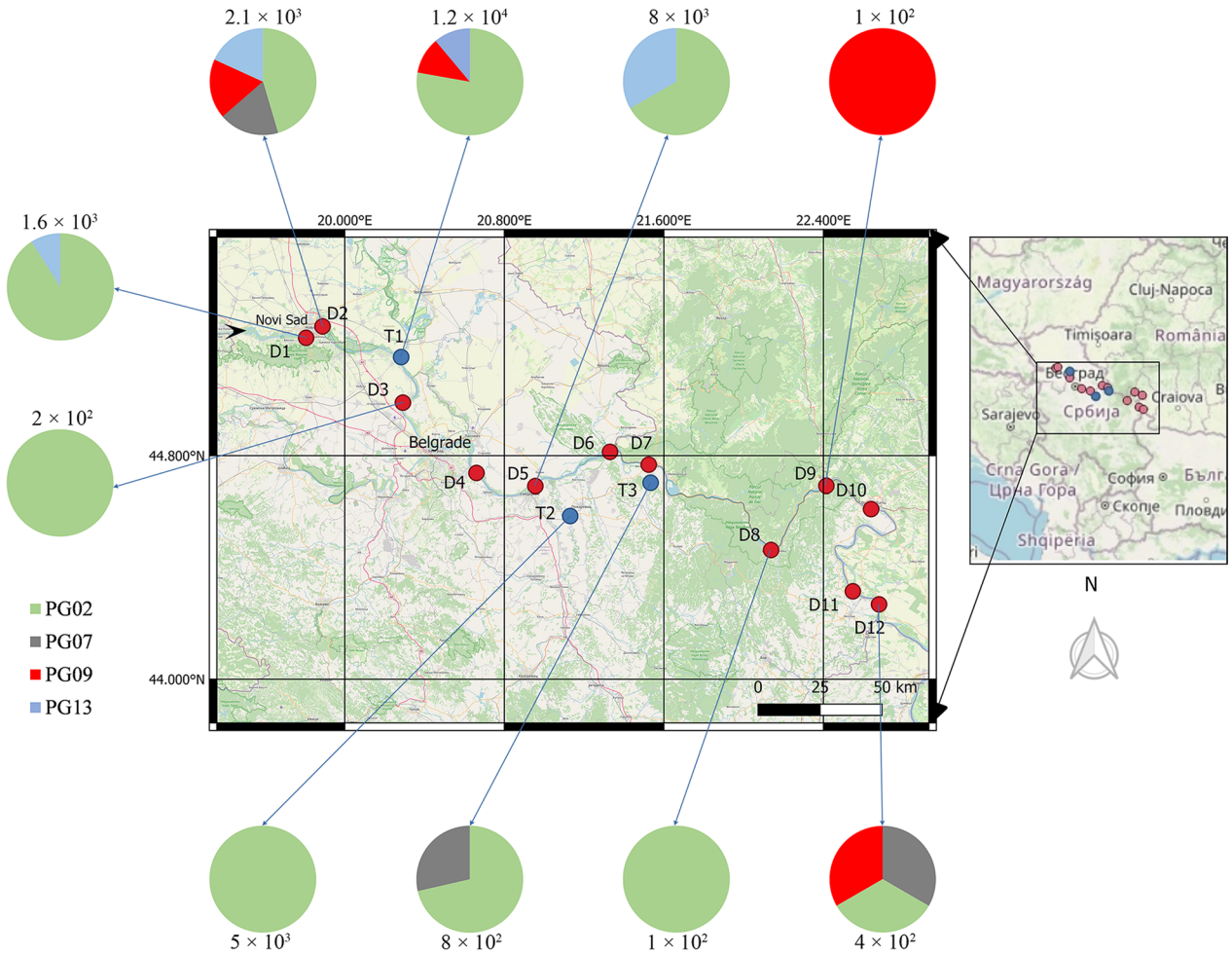

Fig. 1 Sampling locations on the Danube River (D1–D12) and its tributaries (T1–T3). Pie charts associated with sampling sites show the percentage share of detected *Pseudomonas syringae* phylogroups in the total number of isolates from that site. The *Psy* population sizes are indicated next to the pie charts and estimated in CFU per L. The black arrowhead shows the direction of the river flow

(98–100%) with *Psy* sequences from the NCBI nucleotide database, which were compared with query sequences using the blastn algorithm. These 51 isolates were subjected to further phenotypic and phylogenetic characterization. The remaining 28 isolates were excluded from further analysis due to low-quality reads of the partial *cts* gene and percentage identity below 98% similarity with *P. syringae*. Phylogenetic analysis based on partial sequences of the *cts* gene (360 bp) involved 84 sequences (29 reference strains, four strains used as out-groups, and 51 sequences of the isolates from the Danube). The resulting phylogenetic analysis showed that isolates from the Danube belong to phylogroups (PG) 02 (70.6%), 07 (9.8%), 09 (9.8%), and 13 (9.8%) (Fig. 2). Groupings in PGs 02, 07, and 09 had bootstrap values of 77, 79, and 80, respectively, while the bootstrap value for PG13 was

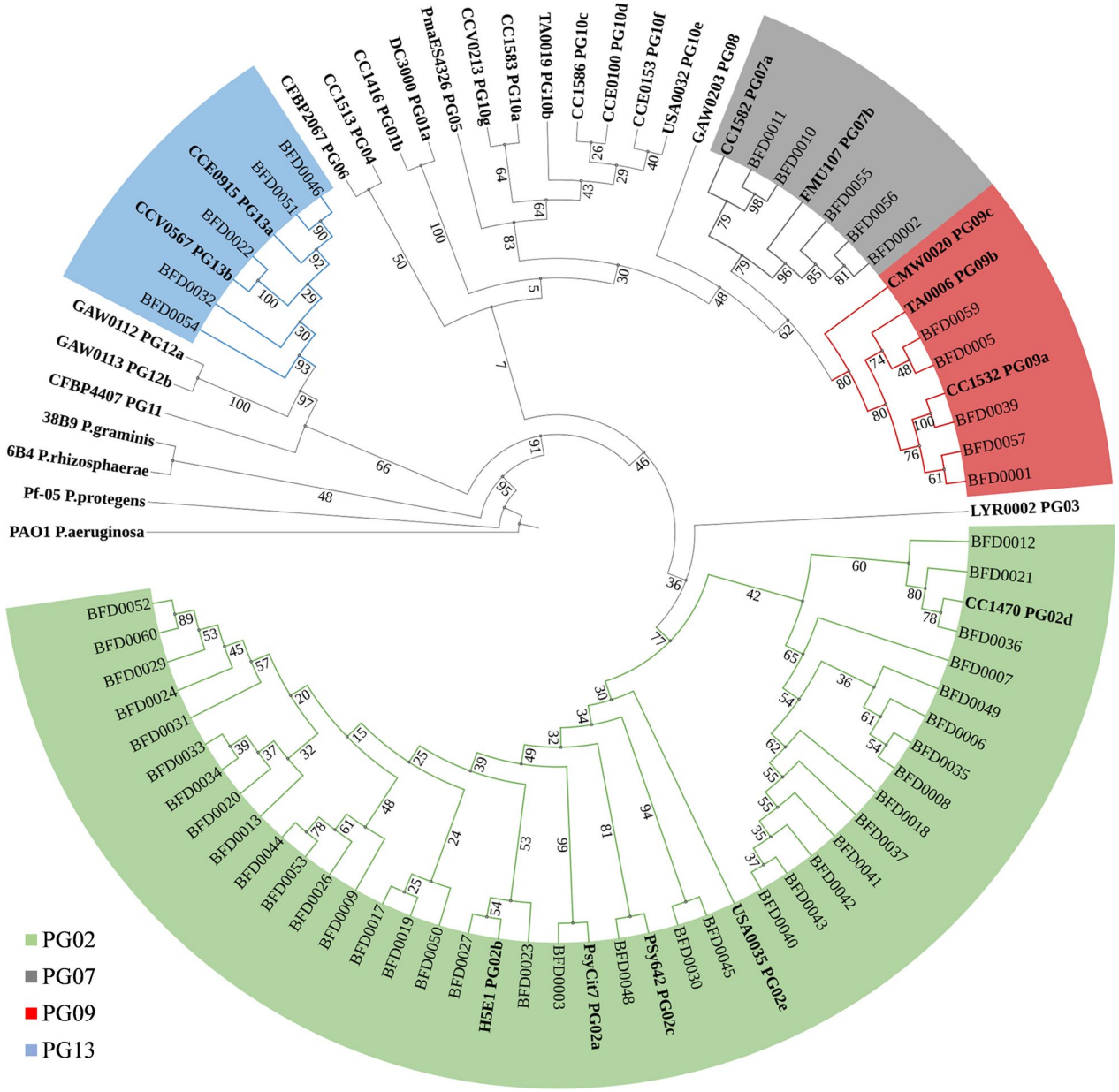

Fig. 2 Phylogenetic tree of *Pseudomonas syringae* isolates based on the partial sequence of the *cts* gene. A phylogenetic relationship between 51 *Pseudomonas syringae* isolates from the Danube (BFD labeled isolates), reference strains representing 13 phylogroups, and strains used as out-groups (in bold) is shown. The tree was generated by the neighbor-joining method. Bootstrap values (expressed as a percentage of 1000 replications) are shown at branch nodes

93. The bootstrap value for clade-level classification in PG07 was 79 for clade 7a and 96 for clade 7b, which is significantly higher than the phylogroup level. Thirty-six out of 51 isolates were representatives of PG02, and these isolates were detected in each locality except Tekija (D9). Isolates BFD0003 and BFD0048 were placed into clades 2a and 2c, respectively, with high bootstrap values of 99 and 81. Five isolates were placed into PG07, PG09, and PG13. Isolate BFD0039 can be considered as a member of clade 9a with a maximum bootstrap value of 100. All tested isolates were distributed among these four *Psy* phylogroups, and there were no representatives of the remaining nine *Psy* phylogroups. Within all collected isolates from the Danube, we encountered 34 different *cts* gene sequences (haplotypes), while the dominant haplotype consisted of sequences belonging to PG02d. We compared the partial sequence of the *cts* gene (360 bp) of the strains detected in this study with those isolated from different diseased crops in Serbia and found 100% similarities between isolates from the Danube and isolates from diseased cherry plants (Ilicic et al. 2021) in Vojvodina Province. This haplotype (strains BFD0018, BFD0035, BFD0037, BFD0041, BFD0042, BFD0043, and BFD0049) was detected at five sampling sites, D1, D2, D3, T1, and T2 in the Danube River Basin (Fig. 1). Moreover, the same haplotype shared 100% identity with strains TAW79 and CC0170 isolated from water samples and cantaloupe in France (Berge et al. 2014; Guilbaud et al. 2016). Another haplotype (isolates BFD0021 and BFD0036) showed 100% similarity to *Psy* strains isolated from diseased cherry plants which are also from Vojvodina Province in Serbia (Balaž et al. 2014), and to strain CC1435 isolated from an epilithic biofilm in France (Berge et al. 2014). In addition, we found high percent similarity (98.89%, four nucleotide differences) of strains BFD0017, BFD0019, BFD0023, and BFD0027 with *Psy* isolates from epidemics on pea and sugar beet in Serbia (Popović et al. 2015a; Nikolić et al. 2018).

Phenotypic characterization

Morphology of the colonies of each isolate grown on King's medium B was described after 24 h of incubation at 30℃ (or after 48 h for slow-growing isolates). Colonies were predominantly small, white or creamy-white, circular, and flat, but within 51 isolates, we encountered orange-yellow colonies (three isolates) and brown pigmented (one isolate). Colony morphology description and the results of other tested phenotypic features are shown in Additional file 1: Table S1.

The pectinolytic activity was confirmed for three isolates (5.88%). Isolates positive for pectinolytic activity were representatives of PG07. The hypersensitive response was induced in *Pelargonium* plants by 35 *Psy* isolates (68.63%) (Fig. 3). 29 isolates of phylogroup 2 (56.86%), three isolates representing PG07 (60%), and

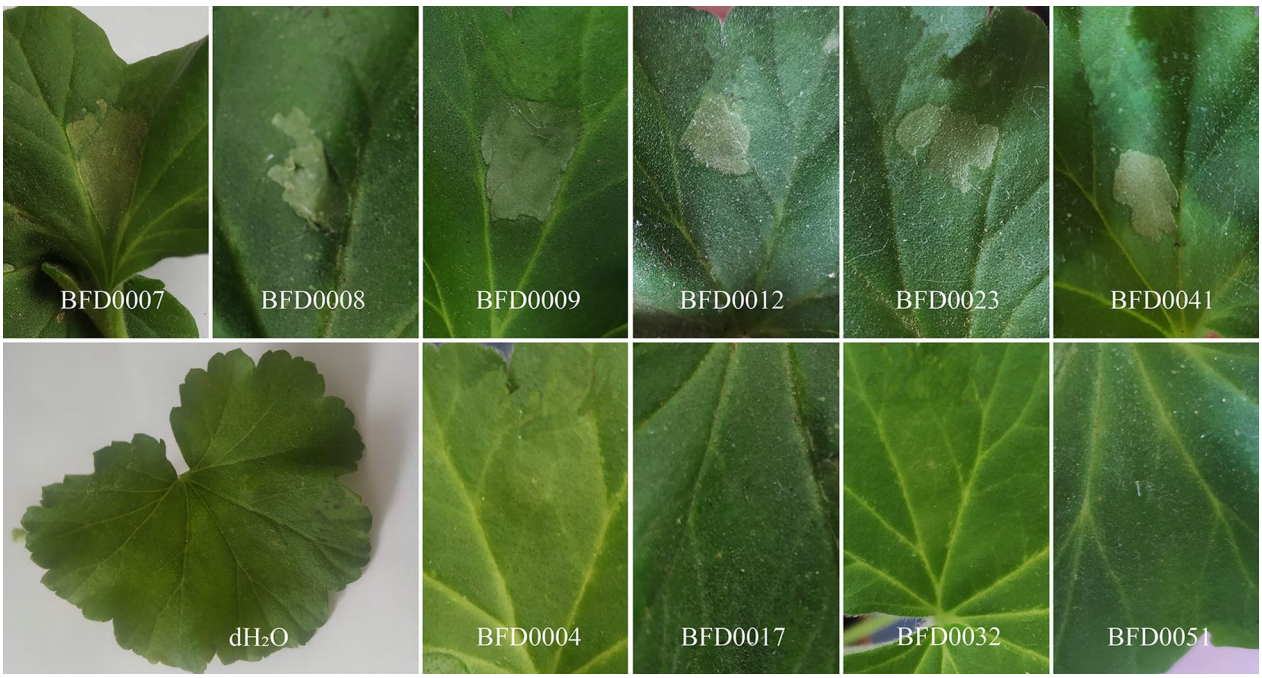

Fig. 3 Hypersensitive reaction assays. Top row, positive reactions induced by *Pseudomonas syringae* isolates from the Danube; bottom row, dH_2O used as a negative control and absence of hypersensitive response

three of PG09 (60%) could induce a hypersensitive response (Additional file 1: Table S1).

Ice nucleation activity

Three different types of ice nucleation activity depending on the temperature of ice formation were observed: warmer than −4°C (type I), −4°C to −7°C (type II), and colder than −7°C (type III) (Joly et al. 2013). Isolates could be classified into ice nucleators type I (7), type II (14), and type III (18), based solely on the average freezing temperatures (Fig. 4). Ice nucleation activity assays resulted in 39 active strains (all three tubes frozen), making up 76.47% of our collection (Fig. 5). INA active isolates from the Danube were from PG02 (94.44% of isolates), PG07 (80% of isolates), and PG09 (40% of isolates). The highest temperature that led to ice formation was −3°C for isolate BFD0052, based on an average value of triplicate tested. There were five more isolates with the capacity to form ice at −3°C (BFD0003, BFD0006, BFD0031, BFD0036, BFD0037), but not in each tested sample. All mentioned isolates that froze water at −3°C belong to PG02. Twelve isolates (BFD0003, BFD0006, BFD0008, BFD0018, BFD0024, BFD0026, BFD0029, BFD0031, BFD0043, BFD0045, BFD0050, BFD0056) cannot be precisely distinguished into categories as the temperature of freezing varies, and it is out of the assigned frame of categories.

Swimming and swarming motility

The swimming area was observed as a circular zone around the inoculation spot and measured using ImageJ software (Additional file 2: Figure S1). Isolates BFD0030, BFD0040, and BFD0048 have shown poor swimming motility. Their growth around the inoculation spot reached 0.36, 0.46, and 0.1 cm^2, respectively. The swimming area formed by the remaining 48 isolates was from 0.56 to 9.35 cm^2. Isolate BFD0055 (representative of PG07) created the most extensive swimming area. *Pseudomonas aeruginosa* PAO1 was used as a positive control, and its swimming zone was 9.23 cm^2. Regarding swarming motility, 25 isolates were swarming-positive. Examples of diverse movement patterns are shown in Additional file 2: Figure S2. The remaining 26 isolates have matched with negative control. All isolates from phylogroups 7 and 9 showed swarming motility. Isolates that were positive on swarming were also representatives of phylogroup 2 (41.67% of isolates) (Fig. 5). The smallest swarming area measured was 3.06 cm^2, formed by isolate BFD0053. Isolates BFD0005 and BFD0001 from PG09 stood out with the biggest swarming areas 59.96 and 59.46 cm^2, respectively.

Discussion

The presence of *P. syringae* in various environmental sources has been known for a long time, while the data about plant isolates still greatly surpasses the data about environmental isolates. Its ability to infect a wide range of hosts contributed to it becoming the best-studied model for understanding plant-microorganism interactions and pathogenicity. There is a great interest in *P. syringae* ecology, epidemiology, and evolution (Xin et al. 2018). They can persist in many habitats like streams, lakes, rivers,

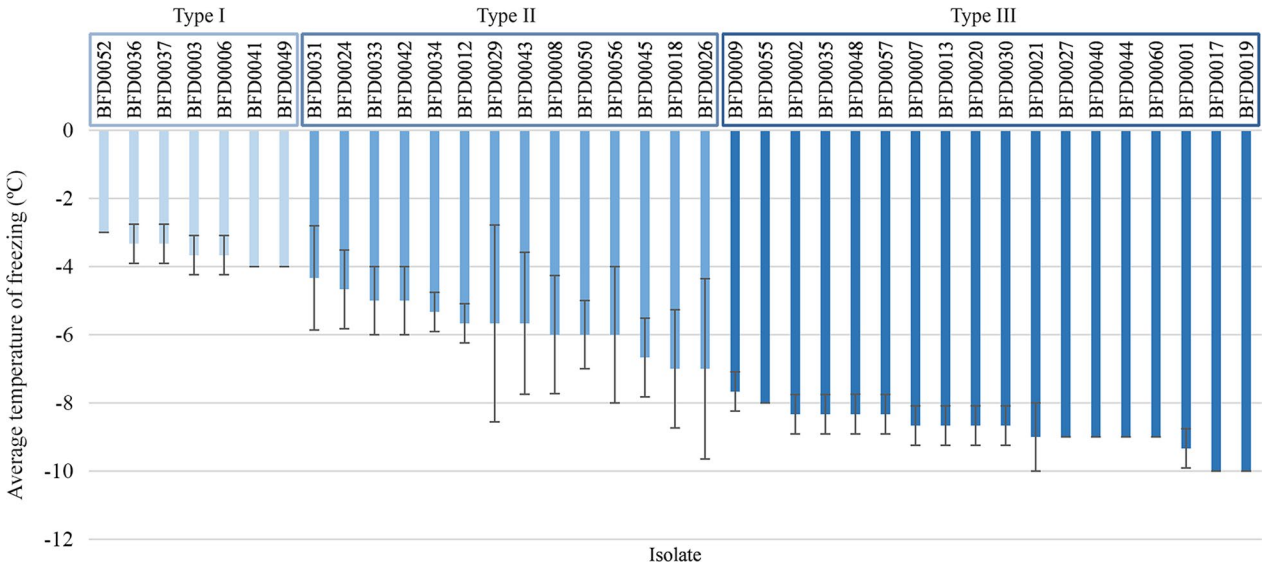

Fig. 4 Average freezing temperature among ice-nucleation positive strains of *Pseudomonas syringae* from the Danube. INA positive strains are grouped into three types based on the average temperature of freezing (Type I < −4°C, Type II −4°C to −7°C, and Type III > −7°C)

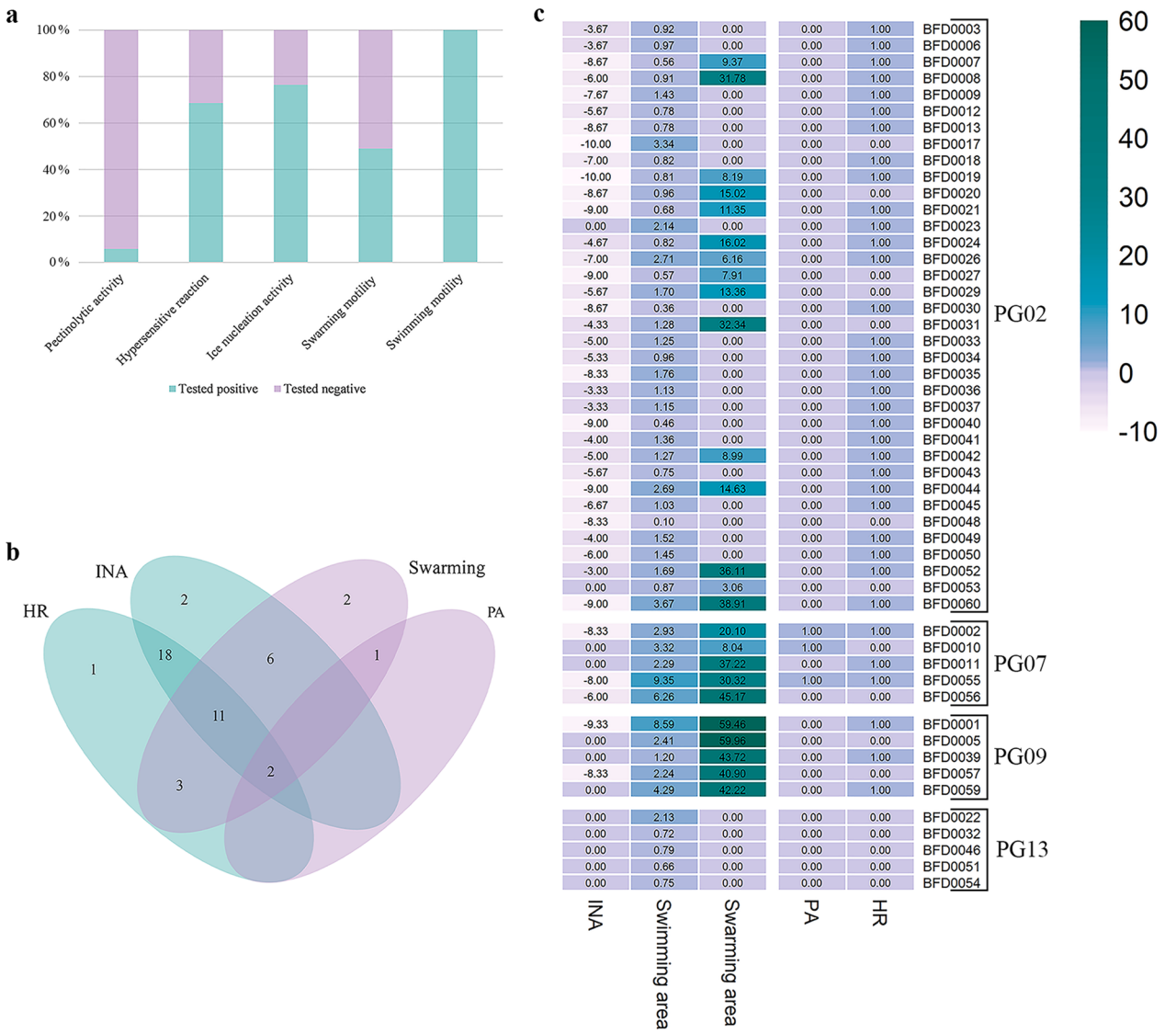

Fig. 5 Phenotypic characterization of *Pseudomonas syringae* isolates from the Danube. **a** The percentage ratio of positive and negative results of phenotypic tests (individual results are provided in Additional file 1: Table S1). **b** Diagram showing the number of isolates sharing the common virulent traits. **c** Heatmap showing gradient in temperature of INA, motility area and capacity to degrade pectin and induce HR for isolates within each phylogroup. *HR* hypersensitive response, *INA* ice nucleation activity, *PA* pectinolytic activity

clouds, rain, and snow, and often lead an epiphytic life on disease-free plants (Morris et al. 2008). All these niches are potential reservoirs of a great diversity of strains and likely serve as sources of newly emerging pathogens (Dillon et al. 2019).

One of our goals was to determine *Psy* abundance in the Danube River. Our results show that the CFU/L of *Psy* ranges from 1.0×10^2 to 1.2×10^4 and that it was present in ten out of 15 sampled sites. Unfortunately, the Danube River in Serbia is affected by pollution from industrial development, agriculture, and poorly treated wastewater (Mănoiu and Crăciun 2021). Because organic material accumulates strongly during filtration of concentrated water samples, we made serial dilutions of the samples, which in combination with the limited filter space possibly affected the range of *Psy* populations, but still allowed us to measure a *Psy* abundance of 10^4 CFU/L. Estimations of the population size of *Psy* isolated from different substrates like wild plants, snow, rain, epilithic biofilms, and lake and stream water can be found (Morris et al. 2008), but there is still insufficient information regarding *Psy* abundance in freshwater habitats. In lake and stream water, the abundance of *Psy* varied from 1.3×10^2 to 1.5×10^4 CFU/L (Morris et al.

2008). Also, in the headwaters of North America, Europe, and New Zealand rivers, the detected abundance of *Psy* was from 50 to 1.0×10^4 CFU/L (Morris et al. 2010). Population densities of *Psy* in the Durance River catchment, where it was monitored during four seasons over 2 years, were up to 1.0×10^5 CFU/L and its presence was confirmed in all sampling sites (Morris et al. 2022). Values of 1.0×10^5 CFU/L were reached during the winter seasons. Still, regarding spring seasons, population densities in the Durance River were lower, which coincides with the results obtained for the Danube River Basin.

The diversity of *Pseudomonas* species along the Danube River was recently assessed, resulting in 611 isolates identified by MALDI-TOF MS. Only two isolates were identified as a *P. syringae,* based on an additional *rpoD* gene sequence analysis (Mulet et al. 2020). The strains were isolated using an incubation temperature of 37°C. The overall diversity was probably much broader than acquired because the incubation and water temperatures during sampling (sampling was performed during late summer) were not optimal and not usually used to isolate environmental *Psy*. In fact, the impact of water temperature on the population size of *Psy* species in freshwater ecosystems has been shown recently, where *Psy* densities decreased with increasing water temperature, and it was the only factor among other aquatic parameters explaining population densities (Morris et al. 2022). Accordingly, the similarity of the occurrence of *Psy* populations in the lower part of the Durance River catchment, which coincides with the sampled sites on the Danube River, and in the Danube River in spring may be a consequence of similar temperature ranges in the same season. The aquatic parameters of the sampled Danube water showed a narrow range of measured values among different sites, and we did not find any correlation between *Psy* abundance and physicochemical parameters. Future seasonal monitoring would provide us with more information on the relationship between *Psy* abundance and variations in aquatic parameters in the Danube.

In the last decade, *Psy*-related disease epidemics on vegetables and fruits caused by *Psy* isolates from PG02 have been detected in the Serbian Danube River Basin. In particular, *Psy* has been identified as a causal agent of diseases in various crops in different parts of Vojvodina Province along the Danube-Tisa-Danube canal network. In particular, affected crops include oil pumpkins with disease incidence (DI) of 5–20% (Balaž et al. 2014), peas where DI reached 10–30% (Popović et al. 2015a), Swiss chard with DI 2–20% (Ignjatov et al. 2015), sugar beet with DI 0.1–40% (Stojšin et al. 2015), carrot, parsley and parsnip with DI 5–20% (Popović et al. 2015b). Among woody plants, its host was cherry, with DI up to 25% (Balaž et al. 2016; Ilicic et al. 2021; Iličić et al. 2022). The highest DI of 80% was recorded in the blueberry orchard in Šabac, where 10% of the plants died (Zlatković et al. 2022). All these reports represent disease occurrence at sites surrounded by the Danube River and the Danube-Tisa-Danube Canal. We reasoned that these freshwater bodies could be a possible source of *Psy* inoculum and consequently a risk for crops in this area. The concordance of *cts* sequences from two haplotypes of *Psy* strains isolated from the Danube River and the causal agents of the reported disease on cherry plants suggests the presence of mixed environmental and agricultural populations and the putative risk of the Danube irrigation system for future disease occurrence. However, long-term monitoring of the Danube River Basin is needed to assess the extent of this risk.

The considerable genotypic and phenotypic heterogeneity of *Psy* species makes it difficult to access the full diversity of this group during isolation (Berge et al. 2014). Phenotypic comparisons with some previously described reference strains may result in the loss of a substantial number of *Psy* group members. A PCR-based method for detecting *Pseudomonas syringae* using *Psy*-specific primers (Guilbaud et al. 2016) is much more comprehensive than isolation based on phenotypic characteristics. It allowed us to cover the entire *Psy* group and isolate different strains that show great phenotypic diversity within the *Psy* collection from the Danube River. In addition, the *cts* gene has previously been found to be one of the most reliable genes that can be used for determining the phylogenetic affiliation of *Psy* strains (Berge et al. 2014). We observed the greatest abundance of *Psy* isolates belonging to PG02 and the highest phylogenetic and phenotypic diversity within this phylogroup among the whole *Psy* isolate collection from the Danube. PG02 is the most ubiquitous group for which numerous isolates have been found in environmental habitats, including water sources (Berge et al. 2014). Additionally, strains from rivers that belong to PG02 are more aggressive on cantaloupe seedlings than strains in other phylogroups (Berge et al. 2014). Isolates from PG02 are known as potential ice nucleators (Pietsch et al. 2017), and previous estimations show that 85% of isolated strains from PG02 were INA-positive (Morris et al. 2010). Members of PG02 isolated from freshwaters have been linked on the basis of their *cts* gene sequences to strains characterized as worldwide causative agents of reported epidemics in apricots, melons, squash, and sugar beets (Morris et al. 2022). Morris et al. (2022) identified the haplotype DD.1 (PG02b) as the most abundant in their *Psy* strain collection from the Durance River catchment, whose partial sequence of the *cts* gene has 100% similarity to sequences of *Psy* previously isolated from numerous disease epidemics worldwide, including sugar beet

epidemics in Serbia. Although we detected 34 different haplotypes based on the *cts* gene in the strain collection from the Danube River Basin, we did not find 100% similarity to the DD.1 haplotype (the highest similarity was 98.89%, four nucleotide differences, in strains BFD0017, BFD0019, BFD0023, and BFD0027). The representative of PG02c, isolate BFD0048, did not elicit a hypersensitive response in the *Pelargonium* plant. Strains from clade 2c are known to be members of the *Pseudomonas congelans* species (Behrendt et al. 2003). Although they have been primarily described as non-pathogenic (Dillon et al. 2019), their involvement in the development of diseases in citrus plants in Tunisia has been reported (Oueslati et al. 2020). In addition, the pathogenicity of *P. congelans* isolated from the phyllosphere of Serbian autochthonous plum cultivars was reported with a severity index of 44% on the leaves (Janakiev et al. 2020).

P. syringae isolates of other phylogroups detected in the Danube River (PG07, 09, and 13) were not often detected as pathogens of diseases in crops in Serbia. To date, there was one initial report of *P. viridiflava* (PG07) in the southern part of Serbia (outside the Danube River Basin) with a disease incidence of 10–25% on tomato plants (Popović et al. 2015c). Strains from this phylogroup have also been previously isolated from water habitats, and they are known as *P. viridiflava* representatives of the *Psy* species complex (Lipps and Samac 2022). Their prominent feature is the production of the enzyme pectate lyase, which is used to degrade pectin in plant cell walls as one of the main virulence factors (Lipps and Samac 2022). Moreover, by an investigation from the beginning of the twenty-first century until 2015, *P. viridiflava* was responsible for 18% of all disease outbreaks caused by the *Psy* species complex (Lamichhane et al. 2015). PG09 strains have been reported exclusively from aquatic habitats and are considered to be well adapted to the environment, while PG13 is also widespread but mostly found on non-plant substrates (Berge et al. 2014).

To get additional insight into the colonization capabilities of *Psy* strains from the Danube which could be considered as an important pathogenic feature, we examined their motility. The ability to move toward nutrients, attach, and penetrate host tissues is an excellent advantage for a pathogenic lifestyle (Colin et al. 2021). Examined forms of motility, swimming and swarming, are flagellum-mediated and depend on environmental conditions (Markel et al. 2018). While swarming is locomotion on a semi-solid surface, swimming occurs in the liquid phase (Jose and Singh 2020). A connection between swarming motility and host range extent was detected earlier, and strains with a tendency to swarm in the first 24 h have been shown to have a broader host range than non-swarming ones (Morris et al. 2019). In our collection, we encountered numerous strains that stand out with their ability to swim and swarm in the given conditions, including strains from PG02, which, according to the data available so far, pose the greatest threat to plants in the Danube River Basin. That suggests that among the *Psy* isolates from the Danube, those with the largest motility areas can pose a threat to plant health because this trait makes them a potential pathogen with increased virulence.

By comparing sequenced genomes of environmental and crop-pathogenic strains and identifying genes with key roles in disease emergence, the hypothesis that crop-pathogenic *P. syringae* diverged from a pre-existing population that was present in the environment before the development of modern agriculture was suggested (Monteil et al. 2016). A significant genetic similarity between strains from nonagricultural habitats and those isolated from disease epidemics was shown (Morris et al. 2008; Monteil et al. 2013). Mixing populations from agricultural and environmental sources is highly possible, bearing in mind that they are genetically almost indistinguishable (Bartoli et al. 2015; Monteil et al. 2016). These data increase the importance of studies that provide insight into the diversity of populations outside of agroecosystems. Regarding quite frequent reports of plant diseases caused by strains from the *Psy* complex (especially PG02), high diversity among *Psy* strains from the Danube River, and extensive use of Danube freshwater for crop irrigation purposes, future studies should be focused on monitoring seasonal population changes and determination of pathogenicity and the host range of the isolates in order to assess the potential risk for irrigation of certain crops and even predict potential epidemics.

Conclusions

This study represents the first report on the abundance and diversity of the plant pathogen *P. syringae* in the Danube River Basin. We revealed the abundance of *Psy* in the Danube River, and the presence of four phylogroups of *Psy*, including isolates with confirmed putative virulent traits such as ice nucleation activity, flagellum-dependent motility, and pectinolytic activity. It remains to be investigated whether the Danube River Basin is a possible source of pathogen inoculum for irrigated crops.

Methods

Sample sites and collection

Samples were collected from 11 to 14th May 2021 from 15 sites (Fig. 1). Sites D1–D12 were situated on the Danube River, while sites T1–T3 were located at the Danube tributaries. In the investigated stretch, the Danube has characteristics of a large lowland river. Except for Site T3 (the River Pek), all sites are heavily modified water bodies

affected by the Iron Gate dams. The construction of the Iron Gate dams resulted in hydromorphological changes in this part of the river and the formation of reservoirs with reduced sediment flux, increased sediment deposition, and slowed river flow (Vuković et al. 2014). All samples were collected approximately 3 m from the shore at a 30 cm depth below the surface in clean, sterile 500 mL bottles. Samples were immediately stored at 4°C and transported to the laboratory in cooling boxes (within 3 h). In-field measurements (temperature, pH, conductivity, and dissolved oxygen levels) were performed using a multi-parameter probe (WTW/Xylem Analytics, Germany). Data on measurements are provided in the Additional file 1: Table S1.

Sample processing and PCR detection of *P. syringae*

Water samples were serially diluted in sterile distilled water (tenfold, 100-fold, and 1000-fold) in the final volume of 100 mL and filtered. Membrane filtration was done using a mixed cellulose esters filter with a pore diameter of 0.45 µm and a filter diameter of 47 mm (Millipore, France). Filters were transferred face up on the surface of KBC media (Mohan and Schaad 1987), selective for the growth of *Pseudomonas* species, and incubated for 3–5 days at room temperature. Since we filtered three different dilutions of sampled river water for abundance estimation and *Psy* identification, we further processed dilutions with a countable number of colonies (100 colonies or less per filter for tenfold dilutions (47 mm diameter filter), except for the D4 site with 145 colonies in 100-fold dilution). In total, 1257 grown colonies from the filters were subjected to a *Psy*-specific PCR reaction to detect *Pseudomonas syringae* species using primers named Psy_F and Psy_R, designed by Guilbaud et al. (2016). PCR reactions were conducted in MiniAmp™ Thermal Cycler (Thermo Fischer Scientific, Waltham, Massachusetts, USA). PCR mixture and conditions used were also described by Guilbaud et al. (2016). In brief, PCR reactions were conducted in a final volume of 25 µL with each mix containing 14 µL of PCR water (Thermo Fischer Scientific, Waltham, Massachusetts, USA), 5 µL of 10× KAPA Taq buffer (KAPA Biosystems Inc, USA), 1.5 µL of $MgCl_2$ (25 mM), 0.3 µL of dNTP mix (2.5 mM per nucleotide), 0.2 µL of KAPA Taq Polymerase (5 U/µL, KAPA Biosystems Inc, USA) and 1 µL of each *Psy*-specific primer (10 mM). Bacterial cell material from single colonies was picked up with a sterile 10 µL pipette tip and transferred directly into PCR tubes with a PCR mixture. PCR reactions were conducted with an initial denaturation step for 5 min at 96°C, followed by 30 cycles at 94°C for 30 s, 61°C for 30 s, 72°C for 30 s, and final elongation for 10 min at 72°C. PCR products were visualized on 1% agarose gel containing an aqueous solution of 10 mg/mL ethidium-bromide (SERVA, Germany). Electrophoresis was run in Tris–borate-EDTA buffer (5.4 g Tris; 2.75 g Boric acid; 4 mL 0.5 M pH 8 EDTA; dH_2O to 1 L) at a constant voltage of 90 V and 300 mA amperage for 60 min. *Psy*-PCR positive strains were stocked in LB medium with 20% glycerol and stored at −80°C.

DNA extraction

For total genomic DNA extraction, the modified CTAB protocol proposed by Le Marrec et al. (2000) was used. Single bacterial colonies of each isolate were resuspended in a mix of 567 µL of TE buffer (pH 7.6; 10 mM Tris; 1 mM EDTA), 30 µL of 10% SDS, and 3 µL 20 mg/mL of proteinase K. After incubation at 37°C for 30 min, 100 µL of 5 M NaCl and 300 µL of 3% CTAB + PVP buffer (pH 8; 3% CTAB; 1 M Tris; 1.4 M NaCl; 20 mM EDTA; 3% PVP) was added, vortexed, and incubated on the heating block at 65°C for 20 min. In the next step, the DNA was purified with 800 µL of chloroform, vortexed thoroughly, and centrifugated for 10 min at 8000 *g*. Supernatant in the upper phase was transferred to a new tube and mixed with 3 M sodium acetate (pH 5) in a volume ratio of 1:10. An equal volume of ice-cold isopropanol (~750 µL) was added and the mix was centrifugated for 15 min at 8000 *g*. The liquid phase was discarded, and the precipitate was washed with 1 mL of ice-cold 96% ethanol and centrifugated for 10 min at 8000 *g*. The liquid phase was discarded again, and the precipitate was dried for 30 min at 37°C and resuspended in 50 µL of TE buffer with RNase mix added in a final concentration of 0.2 mg/mL. The isolated genomic DNA was incubated for 15 min at 37°C and stored at −20°C.

Phylogenetic analysis

To further confirm *P. syringae* identity and their phylogeny, a partial sequence of citrate synthase housekeeping gene (*cts*) was amplified and sequenced. DNA amplification and sequencing were done with primers described previously (Morris et al. 2010). The PCR reaction was done in a final volume of 50 µL containing 25 µL of DreamTaq™ Green PCR Master Mix 2× and 21 µL of PCR water (Thermo Fischer Scientific, Waltham, Massachusetts, USA), 1 µL of each primer (10 µmol) and 2 µL of template DNA. PCR reactions were conducted with initial denaturation for 5 min at 96°C, followed by 35 cycles at 94°C for 30 s, 62°C for 1 min 30 s, 72°C for 2 min, and final elongation for 7 min at 72°C. PCR products were purified with GeneJET PCR Purification Kit (Thermo Fischer Scientific, Waltham, Massachusetts, USA) and Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA). PCR products were visualized on 1% agarose gel containing an aqueous solution of 10 mg/mL ethidium-bromide (SERVA, Germany). Electrophoresis

was run in Tris–borate-EDTA buffer (5.4 g Tris; 2.75 g Boric acid; 4 mL 0.5 M pH 8 EDTA; dH_2O to 1 L) at a constant voltage of 90 V and 300 mA amperage for 90 min for partial *cts* gene (citrate synthase gene) products. Agarose gels were visualized with a transilluminator (LKB, Transilluminator 2011 Microvue UV Light, Sweden). All amplicons were sequenced by Eurofins Genomics GmbH (Wien, Austria) using the sequencing primer described previously (Morris et al. 2010). Sequences were processed using the FinchTV v. 1.4.0 software package (Geospiza Inc.). Sequencing was repeated up to three times for certain samples to avoid sequencing errors and obtain more reliable results. Sequences that had low quality or less than 98% similarity to *Psy* were excluded from phylogenetic analysis. Sequences of representative *P. syringae* strains were retrieved from Berge et al. (2014) and included in the phylogenetic analysis to determine the phylogenetic grouping of *P. syringae* strains isolated from the Danube. Selected reference strains included one representative of each phylogroup and clades within phylogroups. Among reference strains, there was chosen one strain representing PG03 (LYR0002), PG04 (CC1513), PG05 (PmaES4326), PG06 (CFBP2067), PG08 (GAW0203), and PG11 (CFBP4407), two strains representing PG01 (DC3000—clade 1a; CC1416—1b), PG07 (CC1582—7a; FMU107—7b), PG12 (GAW0112—12a; GAW0113—12b), and PG13 (CCE0915—13a; CCV0567—13b), three from PG09 (CC1532, TA0006, CMW0020—clades from a to c, respectively), five from PG02 (PsyCit7, H5E1, PSy642, CC1470, USA0035—clades a to e, respectively) and seven strains representing PG10 (CC1583, TA0019, CC1586, CCE0100, USA0032, CCE0153, CCV01213—clades a to g, respectively). Reference strains *P. graminis*, *P. rhizosphaerae*, *P. protegens*, and *P. aeruginosa* were included as out-groups, as used by Berge et al. (2014). The tree was rooted on the *P. aeruginosa* PAO1 strain. The *cts* gene sequences were aligned using the Muscle program integrated into the Mega 11 software and used to create a dendrogram in Mega 11, inferring evolutionary history using the neighbor-joining method. The percentage of replicate trees where associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. Sequences from this research were deposited in the NCBI database under the following accession numbers: OP620590-OP620592, OP620594-OP620602, OP620606-OP620613, OP620615-OP620616, OP620618-OP620626, OP620628-OP620635, OP620637-OP620646, and OP620648-OP620649. The *cts* gene partial sequences used for comparison among all collected isolates from the Danube were subsequently blasted using the NCBI BLASTn tool in order to assess similarities between sequences and distinguish haplotypes.

Phenotypic characterization

Phenotypic characterization of 51 *Psy* isolates previously confirmed by sequencing of partial *cts* gene included a description of colony morphology, pectinolytic activity, induction of a hypersensitive reaction, ice nucleation activity assay, swimming, and swarming assays.

Besides the isolates from the Danube, other bacterial strains used in phenotypic characterization as controls were: *P. aeruginosa* PAO1, *P. syringae* pv. *aptata* P5, and *Pectobacterium carotovorum* 2811. Strains used in this study were cultivated as overnight cultures in KB broth (30°C with shaking at 180 rpm, except *P. aeruginosa* PAO1 that was incubated at 37°C) or streaked on a solid KB (24 h incubation at 30°C). Depending on the phenotypic test, adjusting optical density was done in fresh KB broth to $OD_{600} \sim 0.2$ (approximately 1.0×10^8 CFU/mL) and $OD_{600} \sim 0.3$ to 0.4 (approximately 2.0×10^8 CFU/mL). Optical density adjustment was made based on the growth curves of two randomly selected isolates from the Danube River. In brief, overnight cultures of the isolates BFD0012 and BFD0052 were diluted 50-fold in 50 mL of fresh KB medium and incubated at 30°C with shaking at 180 rpm. OD_{600} was measured every 45 min. In parallel, samples from the incubated culture were taken at a given checkpoint, serially diluted in 0.01 M $MgSO_4$, and 0.1 mL of dilutions was poured and spread plated onto solid KB medium. After incubation at 30°C for 24 h, the CFU/mL was calculated. Obtained values of optical density and CFU/mL for each checkpoint were utilized for constructing a standardization curve for easy use of optical density to approximate bacterial cell density (Additional file 2: Figure S3). For both isolates, there are visible lag phases and gradual entry in the exponential phase of growth which is followed by the beginning of the stationary phase. For BFD0012, cell density reaches 1.0×10^8 CFU/mL at $OD_{600} = 0.212$ and 2.0×10^8 at $OD_{600} = 0.318$. For BFD0052, cell density was 1.0×10^8 CFU/mL at $OD_{600} = 0.156$ and 2.0×10^8 at $OD_{600} = 0.284$.

Pectinolytic activity

The pectinolytic activity was tested using 1 cm thick potato slices placed in a sterile Petri dish with filter paper soaked in sterile water. 100 µL of bacterial suspensions with OD_{600} adjusted to ~ 0.2 were inoculated into indentations made in potato slices with a sterile 1 mL pipette tip. Each isolate was tested in triplicate. Results were observed after 48 h of incubation at room temperature. Softened, brown tissue around the indentations was a

sign of a positive reaction. The positive control used for this test was the *P. carotovorum* 2811 strain.

Hypersensitive reaction

The hypersensitive reaction was tested on *Pelargonium* plants. Bacterial suspensions were adjusted to $OD_{600} \sim 0.2$, centrifugated for 10 min at 2000 *g*, and resuspended in sterile distilled water. Using a needleless medical syringe, *Pelargonium* leaves were inoculated with bacterial suspensions on the abaxial side between two lateral veins. Each isolate was tested in triplicate. Sterile distilled water was used as a negative control. Results were assessed 24–48 h and 7 days post-inoculation.

Ice nucleation activity assay

Ice nucleation activity was tested using a CH-100 Cooling Dry Block (Biosan, Riga, Latvia) with a protocol modified and optimized according to Nemecek-Marshall et al. (1993) and Joly et al. (2013). From overnight cultures, 2 mL volume was exposed to a temperature of 15°C for 1 h in the stationary growth phase to induce expression of ice nucleation phenotype. After induction, the optical density of isolates was adjusted to $OD_{600} \sim 0.2$ in the final volume of 1 mL in a fresh KB medium. The bacterial suspensions were centrifugated for 10 min at 2000 *g*, and the precipitate was resuspended in the same volume of sterile dH_2O. 20 µL of prepared bacterial suspensions, which contain 2.0×10^6 CFU/mL, was distributed to 0.2 mL PCR tubes, each isolate in triplicate (three tubes). PCR tubes were placed in a Cooling Dry Block, and isolates were exposed to decreasing temperatures from −2°C to −10°C in a decline of 1°C. Samples were checked for ice formation every 5 min (freeze check was performed with sterile tips), an interval of exposition to a given temperature. Sterile dH_2O was used as a negative control. If ice was present in two of three tubes in a given temperature range, the isolate was considered INA+. The temperature at which each of the tubes froze was recorded.

Swimming and swarming assays

For motility assays, the optical density of bacterial suspensions was adjusted to $OD_{600} \sim 0.3$. Swimming media was prepared as 50% KB containing 0.25% agar and swarming media as undiluted KB containing 0.4% agar, as described in Hockett et al. (2013). A swimming assay was performed by stabbing bacterial cell suspension in the center of a swimming plate with a sterile 10 µL pipette tip. For the swarming assay, 3 µL aliquots were inoculated onto the center of a swarming plate. The swimming and swarming motilities were observed 20–24 h after incubation at room temperature. ImageJ software was used to measure swimming and swarming areas (Schneider et al. 2012). *P. aeruginosa* PAO1 strain was used as a positive control for swimming motility (Yang et al. 2018). The negative control for the swarming motility assay was *P. syringae* pv. *aptata* P5 strain (Morris et al. 2019).

Abbreviations

cts	Citrate-synthase
INA	Ice nucleation activity
PG	Phylogroup
Psy	*Pseudomonas syringae*

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00174-0.

Additional file 1. **Table S1.** Abundance and phenotypic characterization of *Pseudomonas syringae* collection from the Danube River and physico-chemical parameters of freshwater samples measured on-site. P, pectinolytic activity; HR, hypersensitive response on *Pelargonium* plants; INA, ice nucleation activity; Swimming and swarming area measured in ImageJ and expressed in cm^2

Additional file 2. **Figure S1.** Swimming area (cm^2) of *Pseudomonas syringae* isolates from the Danube measured with ImageJ software. BFD0026, BFD0040, and BFD0059: selected isolates showing gradation in swimming motility; PAO1: *Pseudomonas aeruginosa* was used as a positive control. **Figure S2.** Swarming area (cm^2) of *Pseudomonas syringae* isolates from the Danube measured with ImageJ software. BFD0008, BFD0024, BFD0029, BFD0039, BFD0042, BFD0052, BFD0057, BFD0060: selected isolates showing swarming motility; C: *Pseudomonas syringae* pv. *aptata* P5 was used as a negative control. **Figure S3.** Growth curves of two *Pseudomonas syringae* isolates from the Danube River. **a** *Pseudomonas syringae* BFD0012 OD_{600} and CFU/mL growth curve and standard growth curve. **b** *Pseudomonas syringae* BFD0052 OD_{600} and CFU/mL growth curve and standard growth curve.

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Authors' contributions

IN, TB, and SS designed the research. SK provided water samples for the research and initial sample processing. MA and IR carried out the experiments. MA wrote the first draft of the manuscript, and IR, OM, SK, TB, SS, and IN contributed to the final version. All authors read and approved the submitted version of the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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