Pseudomonas viridiflava, a Multi Host Plant Pathogen with Significant Genetic Variation at the Molecular Level

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Abstract

The pectinolytic species Pseudomonas viridiflava has a wide host range among plants, causing foliar and stem necrotic lesions and basal stem and root rots. However, little is known about the molecular evolution of this species. In this study we investigated the intraspecies genetic variation of P. viridiflava amongst local (Cretan), as well as international isolates of the pathogen. The genetic and phenotypic variability were investigated by molecular fingerprinting (rep-PCR) and partial sequencing of three housekeeping genes (gyrB, rpoD and rpoB), and by biochemical and pathogenicity profiling. The biochemical tests and pathogenicity profiling did not reveal any variability among the isolates studied. However, the molecular fingerprinting patterns and housekeeping gene sequences clearly differentiated them. In a broader phylogenetic comparison of housekeeping gene sequences deposited in GenBank, significant genetic variability at the molecular level was found between isolates of P. viridiflava originated from different host species as well as among isolates from the same host. Our results provide a basis for more comprehensive understanding of the biology, sources and shifts in genetic diversity and evolution of P. viridiflava populations and should support the development of molecular identification tools and epidemiological studies in diseases caused by this species.

Introduction

Pseudomonas species are ubiquitous bacteria endowed with metabolism that enables them to dwell in a large variety of environmental niches. Various Pseudomonas species are important as pathogens of animals, insects and plants [1–3]. The molecular taxonomic criteria for the genus Pseudomonas have been revised along with the progress in bacterial taxonomy. However, due to the inability of DNA-DNA hybridization and 16S rDNA-based methods to reveal intraspecies variability, Yamamoto and colleagues suggested that a phylogenetic analysis using the nucleotide sequences of the housekeeping genes for the beta subunit of the DNA gyrase (gyrB) and σ70 RpoD protein subunit of RNA polymerase (rpoD), which evolve much faster than rDNAs [1], provide the higher resolution necessary for intraspecies variation analysis than 16S rDNA sequences [4].

Traditionally, the phytopathogenic oxidase-negative fluorescent Pseudomonads have been grouped into two species, Pseudomonas syringae and Pseudomonas viridiflava [5]. The LOPAT determinative tests (L: levan production; O: oxidase production; P: pectinolytic activity; A: arginine dihydrolase production; and T: tobacco hypersensitivity) are the most widely used protocol for the differentiation of plant pathogenic Pseudomonads [6,7].

The pectinolytic species P. viridiflava (Burkholder) Dowson, [8,9] has a wide range of hosts causing necrotic leaf and stem lesions and basal stem and root rots. It was originally isolated from the dwarf or runner bean, in Switzerland (reference strain P. viridiflava ATCC13225). However, based on 16S rDNA analysis, P. viridiflava had been placed previously in the P. syringae group [10]. Likewise, following ribotypical analysis, strains of Pseudomonas syringae pv. ribicola (infects Ribes aureum) and Pseudomonas syringae pv. primulae (infects Primula species) were also incorporated into the P. viridiflava species [11].

P. viridiflava is a multihost pathogen causing severe damages to tomato (Solanum lycopersicum) [12,13], melon (Cucumis melo) [14,15], blite (Amaranthus blitum), chrysanthemum (Chrysanthemum morifolium), eggplant (Solanum melongena) [15], and the model plant species Arabidopsis thaliana [16]. Typical symptoms of P. viridiflava infection in tomato are a general wilting and yellowing of the plants and brown-black spots developing at the pruning sites of the stem. In the inner part of the stem, pith and vascular tissues display brown discoloration and soft rot often develops. It is a significant pathogen in the eastern Mediterranean region and Aegean islands in particular, representing 12% and 50%, respectively, of the Pseudomonas species causing stem necrosis [17,18].

The aim of the present study was to evaluate the genetic variation among local and global isolates of P. viridiflava. Several strains from laboratory collections and new isolates from several plant species were studied by a) biochemical markers, b) pathogenicity profiling, c) molecular fingerprinting and d) partial sequencing of the housekeeping genes gyrB (DNA gyrase beta subunit), rpoD (RNA polymerase σ70 subunit) and rpoB (RNA polymerase beta subunit) which have been used assignatures for bacterial identification, as well as loci for phylogenetic analysis.
[19]. To our knowledge, this is the first report worldwide of *P. viridiflava* being a pathogen on *Acanthus mollis* and capitulum bracts of *Cynara scolymus* L.

**Results**

**Biochemical Profiling**

On the basis of their colony morphology, physiological, biochemical, and pathological characteristics, representative isolates of *Pseudomonas* spp. were identified as *P. viridiflava* based on the determinative schemes as proposed previously by various researchers [7,20,21]. Eighteen local (Crete, Greece) isolates were chosen (Table 1) for further characterization, using the LOPAT tests, together with the reference strain *P. viridiflava* NCPPB1249 and other fluorescent *Pseudomonas* species (Tables 1 and 2). Supplementary rapid identification of isolates was achieved by using the pattern of fluorescence on single carbon source media (Sucrose: [−], Erythritol: [+] and DL-Lactate: [+] as described in [22]. All tested isolates gave identical results in these tests as well as in the biochemical profiling to the *P. viridiflava* reference strain and were clearly differentiated from the other fluorescent *Pseudomonas* species (Tables 3 and Table S2). A unique exception was seen in the L(+) Tarrtrate utilization test in which only the tomato isolates tested positive, in contrast to the type strain (Table 3 and Table S2) and the local isolates from other hosts. Thus, the results of the biochemical identification tests did not indicate any variability among the local *P. viridiflava* isolates examined, with the above mentioned exception.

**Pathogenic Profiling and Disease Symptomatology**

Similarly to the biochemical profiling, all *P. viridiflava* local isolates examined had identical pathogenicity profiles when tested on a series of experimental host species (Table S1). Successful inoculations were made on tomato, eggplant, blite, melon, celery, artichoke, acanthus and chrysanthemum under greenhouse conditions. In each host, the symptoms induced by a strain were similar to those caused by each *P. viridiflava* isolate on its natural host (Figure S1). In other words, each isolate induced the same disease symptoms independently of host of origin. On tomato, eggplant, blite, melon, celery and acanthus leaves, the disease started as a water-soaked spot which developed in 3–4 days into small or larger irregular lesions, usually with chlorotic halos. The centre of the lesions later became dry and tan to black in colour. Later the lesions usually coalesced and leaves appeared blighted. Tomato and chrysanthemum plants that were stab-inoculated into the stem developed yellowing in the lower leaves, wilting, and a yellow to brown discoloured pith within 6–10 days. The stem often became hollow and split with bacterial slime exudating. On artichoke, the disease started as water-soaked and dark-green spots on the capitulum bracts. Infected leaves developed sunken and elongated necrotic lesions with a brown to black centre surrounded by thin water-soaked halos along with large dark red-brown margins.

Re-isolations made from the artificially infected plants yielded pure cultures that were confirmed as *P. viridiflava* by LOPAT tests. All local isolates of *P. viridiflava* regardedless of their original hosts (Table 1) caused rust-coloured lesions within 48 h on excised snap bean pods, induced soft rots on pear and did not produce the deep black necrotic pit symptoms on detached lemon fruits [13,15] (Figure S2). The results of the pathogenicity profiling also did not reveal any variability among the *P. viridiflava* strains under study on the plants used for experimental inoculations. However, a validation of the present results against a broader host sampling scheme and detailed phytopathological characterization (e.g. estimation of pathotypes, race specificity, etc.), may provide more relevant information about the intraspecific level of variation of *P. viridiflava* isolates studied.

**Molecular Fingerprinting**

To further investigate inter-strain variability of the local *P. viridiflava* isolates (Table 1), we utilized BOX- (mosaic repetitive sequences of dyad symmetry within intergenic regions), and ERIC- (Enterobacterial Repetitive Intergenic Consensus) like DNA sequences corresponding to conserved repetitive bacterial motifs (collectively known as rep-PCR) to generate genomic fingerprints [23–25]. Rep-PCR fingerprinting is a useful and reliable technique to assess bacterial diversity at the species, subspecies, or even isolate level; its applications to environmental microbiology have been reviewed [26]. This method has high capacity to snap-shot the whole genome, showing greater discriminatory power than PFGE (Pulsed-Field Gel Electrophorese) and MLST (Multilocus Sequence Typing) [27], in comparison with various other phylogenetic methods in bacterial typing and phylogeny [27–32].

The rep-PCR amplifications on total DNA of the eighteen *P. viridiflava* strains showed 9–18 bands in the case of BOX-PCR (Figure 1), and 10–20 bands in the case of ERIC-PCR. A total of 16 discrete bands were scored in both fingerprinting methods, ranging in size from 0.15 kb to 2.6 kb. The data matrix showing presence or absence of the scored bands was analysed with the Jaccard’s coefficient and a combined BOX and ERIC dendrogram [33–35] was created with UPGMA (Figure 2A). All isolates were clustered in two distinct major clusters. The first cluster (Figure 2A; cluster I) included isolates from tomato, eggplant, blite, acanthus and artichoke while the second cluster (Figure 2A; cluster II) contained only the celery isolates (Figure 2A; celery groups 1 and 2).

The first cluster showed the greatest variability and was further divided into three sub-clusters which correlated with the host of origin (Figure 2A; Cluster I). One sub-cluster consisted of the tomato isolates (Figure 2A; tomato group) which were similar to the blite and melon isolates. The second sub-cluster consisted of the eggplant and acanthus isolates. In the third sub-cluster the isolates from artichoke were grouped. The analysis linked closely all strains isolated from the same host, indicating a common genetic base. More specifically, in the eggplant-acanthus sub-cluster, the isolates PV3006, PV570 and PV574a had identical genetic base. More specifically, in the eggplant-acanthus sub-cluster, the isolates PV3006, PV570 and PV574a had identical fingerprinting profiles, while the strain PV3005, isolated from eggplant, was clearly differentiated. The tomato isolates PV441 and PV442 had the same fingerprint but were slightly different from the TKK615 isolate.

The second major rep-PCR cluster was also divided in two sub-clusters with a remarkably high bootstrap value (84%), indicating genetic variability among the isolates from the same host plant (celery). These results led us to conclude that BOX- and ERIC-PCR seem to be able to identify the genetic variability at the intra-species level among *P. viridiflava* isolates, with only few exceptions.

**Phylogeny based on gyrB, rpoD and rpoB gene sequences**

Further analysis of inter-isolate variability was carried out on nucleotide sequences of three PCR-amplified housekeeping gene regions (*gyrB* 840 bp, *rpoD* 615 bp and *rpoB* 1250 bp; total sequence length 2705 bp) for the eighteen local *P. viridiflava* isolates (GenBank accession numbers are given in Table 4 and Table S3), using the Jaccard coefficient. The UPGMA trees generated gave a very good fit when checked by the Mantel test [27](0.94628, 0.97996 and 0.83253 for *gyrB*, *rpoD* and *rpoB*, respectively). Furthermore, the basic topologies were preserved in
the generated trees (Figure S3A), with the \textit{gyrB} sequence tree providing higher resolution in the final consensus tree obtained with the combined sequences of all three genes (Figure 2B).

In general, the Jaccard’s coefficient created the two major clusters seen with the BOX-ERIC tree and grouped the cereal genotypes into two subgroups, although somewhat differently (Figure S3; celery groups 1 and 2). One subgroup consisted of the genotypes PV273a, PV273 and PV271. Theoretically, the influence of stochastic drift on the rate of evolution could be eliminated from the molecular phylogeny.

When the \textit{gyrB} gene sequence was implemented, the constructed tree (Figure S3C) was much more similar to the \textit{spaD} tree rather than to the \textit{gyrB} tree, preserving the general qualitative characteristics of the former. Only one celery sub-group was clearly created, which contained the isolates PV276, PV272a and PV272, PV274, and the second subgroup of the genotypes PV273a, PV273 and PV271. Nevertheless, only the \textit{gyrB} phylogeny separated the celery subgroups from the rest of the genotypes, linking them in a major cluster (Figure S3A; cluster II) which comprised only celery isolates. Another noticeable difference between the \textit{gyrB} and \textit{spaD} trees was that in the \textit{spaD} tree the tomato isolate TKK615 did not group with the rest of tomato isolates (PV441 and PV442, Figure S3B), as was the case in the \textit{gyrB} tree but was placed closer to eggplant, acanthus, and artichoke isolates (Figure S3A, B).

Table 1. Bacterial strains used in this study for biochemical characterization and pathogenicity tests.

| Code          | Host                  | Disease/symptoms       | Location       | Origin               |
|---------------|-----------------------|------------------------|----------------|----------------------|
| PV271         | Apium graveolens L.    | Celery leaf blight     | Heraklion, Cret | This study           |
| PV272         | Apium graveolens L.    | Celery leaf blight     | Heraklion, Cret | This study           |
| PV272a        | Apium graveolens L.    | Celery leaf blight     | Heraklion, Cret | This study           |
| PV273         | Apium graveolens L.    | Celery leaf blight     | Heraklion, Cret | This study           |
| PV273a        | Apium graveolens L.    | Celery leaf blight     | Heraklion, Cret | This study           |
| PV274         | Apium graveolens L.    | Celery leaf blight     | Heraklion, Cret | This study           |
| PV276         | Apium graveolens L.    | Celery leaf blight     | Heraklion, Cret | This study           |
| PV612         | Cucumis melo cv Naudin| Cantaloupe leaf spot/necrosis | Tympaki, Cret | [13]                 |
| PV527         | Amaranthus blitum L.   | Blite (purple amaranth) leaf spot | St. Pelagia, Cret | [13]                 |
| PV3005        | Solanum melongena L.   | Eggplant leaf spot     | Ierapetra, Cret | [13]                 |
| PV3006        | Solanum melongena L.   | Eggplant leaf spot     | Ierapetra, Cret | [13]                 |
| PV570         | Acanthus mollis L.     | Bear’s Breeches leaf blight | Heraklion, Cret | This study           |
| PV574a        | Acanthus mollis L.     | Bear’s Breeches leaf blight | Heraklion, Cret | This study           |
| TK6615        | Solanum lycopersicum   | Tomato spot on fruit   | Antiskari, Cret | [15]                 |
| PV441         | Solanum lycopersicum   | Tomato stem soft rot, pith necrosis | Tympaki, Cret | [13]                 |
| PV442         | Solanum lycopersicum   | Tomato stem soft rot, pith necrosis | Tympaki, Cret | [15]                 |
| PV608         | Cynara scolymus L.     | Artichoke bracts leave lesions/necrosis | Heraklion, Cret | This study           |
| PV609         | Cynara scolymus L.     | Artichoke bracts leave lesions/necrosis | Heraklion, Cret | This study           |
| NCPPB1249     | Chrysanthemum morifolium| Stem soft rot          | United Kingdom (1962) | [15]                 |
| P. savastanoi pv. savastanoi | Ps.sav1 | Olea europaea          | Olive knot disease | Heraklion, Cret | This study           |
| P. savastanoi pv. savastanoi | Ps.sav4 | Olea europaea          | Olive knot disease | Heraklion, Cret | This study           |
| P. savastanoi pv. savastanoi | Ps.sav5 | Olea europaea          | Olive knot disease | Heraklion, Cret | This study           |
| P. syringae pv. tomato | Pst1  | Solanum lycopersicum   | Tomato bacterial speck disease | Tympaki, Cret | This study           |
| P. syringae pv. tomato | Pst2  | Solanum lycopersicum   | Tomato bacterial speck disease | Tympaki, Cret | This study           |
| P. syringae pv. lachrymans | Ps110 | Cucumis sativus        | Angular leaf spot of Cucurbits | Ierapetra Cret | [13]                 |
| P. syringae pv. lachrymans | Ps119 | Cucumis sativus        | Angular leaf spot of Cucurbits | Ierapetra Cret | [13]                 |
| P. syringae pv. syringae | Ps1102 | Cucumis melo          | Angular leaf spot of Cucurbits | Lasithi Cret | [13]                 |
| P. syringae pv. syringae | Ps11 | Citrus lemon           | Citrus blast disease, black pit | Fodele Cret | [13]                 |
| NCPPB2778     | Pyrus communis         | Pear blossom blast and canker | France (1965) | [13]                 |

doi:10.1371/journal.pone.0036090.t001

The phylogeny separated the celery sub-group were mixed with those of strains isolated from melon (PV612) tomato (PV441, PV442) and blite (PV527).
the gyrB, rpoD and rpoB genes. A UPGMA dendrogram was reconstructed and is presented in Figure 2B. The topology of the tree from the combined gene sequences follows the phylogeny of the gyrB and rpoD genes.

The third step in our analysis was to examine the linkage between the local P. viridiflava gyrB, rpoD and rpoB gene sequences along with those deposited in GenBank (Tables 4, S3 and the resulting trees are shown in Figures 3, 4 and 5 respectively). These results corroborated our observations concerning the genetic polymorphism among the P. viridiflava isolates. Furthermore, our results did not reveal any host-specific clustering pattern for the local or deposited strains, since we observed genetic variability even among strains isolated from the same host plant. However, in the gyrB tree (Figure 3), the celery isolates formed a consistent phylogenetic cluster divided into two sub-clusters. It is also noteworthy that four P. viridiflava isolates from A. thaliana (RT228, KNOX249, KNOX753 and DUD6.3a) were clustered together, forming a separate cluster, which was referred to as clade B by Goss and colleagues [16], while all the local isolates seem to be included in clade A [16]. In this dendrogram, the majority of the local isolates fell into two sub-clusters. The first two sub-clusters contained all the celery isolates (PV271 to PV276; Figure 3 celery groups 1 and 2), while the third sub-cluster contained the eggplant (PV3005, PV3006), acanthus (PV570, PV574a) and the artichoke (PV608, PV609) isolates. However, the local isolates from tomato (TKK615, PV441, PV442), blite (PV527) and melon (PV612) were not included in any of the three abovementioned sub-clusters. These local isolates were closely linked to the P. viridiflava reference strains that originated from bean (PDDCC2848 and CFBP2107), A. thaliana (SL243.1b, SL2501b), Cerastium vulgatum (ME751.1a), Draba verna (ME753.1a) and Cardamine parviflora (ME756.1a) (Figure 2). Nevertheless, the topology of the celery isolates in this dendrogram follows the topology described for the local isolates (Figure S3A).

Similarly, another dendrogram was created utilizing the rpoD gene sequences deposited in GenBank (Figure 4). However, the rpoD sequences deposited in GenBank were considerably fewer than those for gyrB. In the rpoD tree, almost all the local P. viridiflava isolates were scattered yet forming three main groups. The first group contained half of the local isolates (local cluster) including all the tomato, eggplant, acanthus and artichoke isolates, while the two others hosted the local celery isolates (celery groups 1 and 2). However, two of the local isolates, (PV3005 from blite and PV612 from melon) were grouped separately from all the other local isolates. The blite isolate was grouped with strain BC2506 originating from Brassica napus, while the melon isolate was grouped with strains originating from bean and Ribes aureum (BFBP2107, PDDCC2848 and NCPPB 963 respectively).

The dendrogram constructed from the rpoB sequences deposited in GenBank (Figure 5) revealed even less information due to the lack of deposited sequences. Nevertheless, findings from this analysis appear to be similar to those derived from the analysis of gyrB and rpoD. No host-specific clustering pattern emerged, even in the case of celery isolates. Although these isolates were grouped again in two groups, they did not appear to be closely linked. In this rpoB-derived phylogeny, some of the local isolates appeared closely linked yet had a scattered pattern and were separated from the rest of the isolates. However, this may be merely an artifact due to the small number of publicly deposited sequences.

**Table 2.** LOPAT tests of eighteen local (Crete, Greece) P. viridiflava isolates along with P. viridiflava reference strain NCPPB1249 and other pseudomonads.

| Species          | Strain No | Levan | Oxidase | Potato rot | Arginine | Tobacco (HR) | Fluorescence pigment |
|------------------|-----------|-------|---------|------------|----------|--------------|----------------------|
| Pseudomonas viridiflava | PV271     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV272     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV272x    | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV273     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV273x    | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV274     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV276     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV612     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV527     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV3005    | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV3006    | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV570     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV574a    | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | TKK615    | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV441     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV442     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV608     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | PV609     | -     | -       | +          | -        | +            | +Blue                |
| Pseudomonas viridiflava | NCPPB1249 | -     | -       | +          | -        | +            | +Blue                |
| P. syringae       | All strains | +    | -       | -          | -        | +            | Green-Blue           |
| P. savastanoi    | All strains | +    | -       | -          | -        | +            | Green                |

doi:10.1371/journal.pone.0036090.t002
| Solanum lycopersicum | Solanum melongena | Apium graveolens | Amaranthus blitum | Cynara scolymus & Acanthus mollis | Cucumis melo | P. viridiflava NCPPB1249 | Other Pseudomonas species |
|----------------------|-------------------|-----------------|------------------|---------------------------------|-------------|--------------------------|---------------------------|
| Tests                |                   |                 |                  |                                 |             |                          |                           |
| Levan                | –                  | –               | –                | –                               | –           | +                        | +                         |
| Oxidase              | –                  | –               | –                | –                               | –           | –                        | –                         |
| Potato rot           | +                  | +               | +                | +                               | +           | –                        | –                         |
| Arginine dihydrolase | –                  | –               | –                | –                               | –           | –                        | –                         |
| Hypersensitivity     | +                  | +               | +                | +                               | +           | +                        | +                         |
| Nitrate reduction    | –                  | –               | –                | –                               | –           | –                        | –                         |
| Fluorescent pigment  | +                  | +               | +                | +                               | +           | +                        | +                         |
| Gelatin hydrolysis   | +                  | +               | +                | +                               | +           | –                        | –                         |
| Pectate gel pitting$^2$ | +                | +               | +                | +                               | +           | NT                       | NT                        |
| Lipases              | +                  | +               | +                | +                               | +           | NT                       | NT                        |
| 2-Ketogluconate      | –                  | –               | –                | –                               | –           | –                        | NT                        |

$^2$ = positive; (–) = negative; NT = not available. Information for additional biochemical experiments can be found in supplementary Table S2. doi:10.1371/journal.pone.0036090.t003
Discussion

*P. viridiflava* is distinguished from many other plant pathogens in being able to infect a large variety of host species including the model plant *A. thaliana* [16]. This presumably reflects a greater degree of hidden genetic variability and is of great interest because it provides a basis to understand how *P. viridiflava* infect different plant/tissues, and could support the development of tools for disease control and management. Also, *P. viridiflava* is often reported as an opportunistic pathogen [13] and thus could experience selection pressures during the epiphytic phase of its life history that are less prevalent in single-host pathogens [16].

*P. viridiflava* has a broad distribution following no particular geographic map structure. Furthermore, variation among *P. viridiflava* isolates from specific hosts appears to be equivalent to the variation among isolates from different hosts, at least for most of the hosts [16]. Goss and colleagues suggested that *P. viridiflava* is not adapted specifically to any host plant species at the local level, since the genetic variation observed within *Arabidopsis* isolates follows the genetic variation also observed in a global sample of isolates from different hosts. This stands in contrast to studies with related plant pathogenic bacteria, which generally show either little variation [36] or high levels of geographically structured variation [37]. Furthermore, a worldwide sample of *P. syringae* pv. *tomato* and *P. syringae* pv. *maculicola* showed unique fingerprints for almost all isolates [38].

In this study, we examined the patterns of genetic variation among *P. viridiflava* isolates collected from various host plants growing in various areas of the island of Crete (South Greece). Although there is a growing interest in elucidating the population structure and genetic variation in many plant pathogenic bacteria, there is limited data available in the case of *P. viridiflava*. The genetic polymorphism of *P. viridiflava* isolates from Crete was determined by rep-PCR (BOX and ERIC) as well as by the partial *gyrB*, *rpoD* and *rpoB* gene sequence and, phenotypic profiling by pathogenicity and biochemical tests. The pathogenicity screens and biochemical profiling did not reveal any polymorphism among the isolates examined and thus did not enable us to further study the genetic variability of the local *P. viridiflava* isolates.

However, the ability of rep-PCR for snapshotting the whole bacterial genomes makes it ideal for intraspecific population analyses as previously described for other species [29,39]. Furthermore, the sequencing of specific genomic fragments was employed for further investigation of the population variability. Although analysis of 16S rDNA sequence is frequently used, the degree of resolution obtained is not sufficient to reveal the real intraspecific relationships because of the extremely slow rate of rDNA evolution [1]. As previously reported, the 16S rDNA-based phylogeny, derived from a single gene, does not necessarily represent the phylogeny of the organisms [40]. Thus, we chose to develop phylogenies based on three housekeeping genes, *gyrB*, *rpoD* and *rpoB*, which have been shown to be useful in grouping isolated strains of several bacterial species and has been extensively used previously [41,42]. The *gyrB*, *rpoD* and *rpoB* partial sequences in

![Figure 1. Agarose gel electrophoresis of BOX-PCR of 18 local *P. viridiflava* isolates.](image1)

![Figure 2. Phylogenetic trees of the local *P. viridiflava* isolates.](image2)
combination with the results obtained from the rep-PCR amplification enabled us to investigate the diversity in the populations of *P. viridiflava*. Because these genes evolved much faster than 16S rDNA, they provide higher resolution in dendrogram generation [43].

Our analysis revealed that the *gyrB* phylogenetic tree for the local isolates was topologically almost identical to the tree based on the rep-PCR fingerprinting, while the phylogenetic tree based on the *rpoD* and *rpoB* gene sequences revealed clearly different patterns of variation (Figures 2A and S3). It is noteworthy that the *gyrB* sequence used for our analysis includes 36 parsimony informative positions (a site is parsimony-informative if it contains at least two types of nucleotides, and at least two of them occur with a minimum frequency of two), while the *rpoD* and *rpoB* sequences had 27 and 14 respectively. This indicates that the *gyrB* gene sequence is more informative for phylogenetic studies and intra-species genetic variability of *P. viridiflava*, a fact that has also been stated previously for other bacterial species [1].

However, as previously reported [16], the combination of several individual sequence fragments in phylogenetic tree generation results in significant alterations in the associations among isolates compared to those in the trees derived for the individual loci. In our case, the phylogenetic trees obtained from the combined *gyrB*, *rpoD* and *rpoB* sequences showed substantial loss in substructure compared to trees generated for the individual loci. As proposed by Goss et al. [16], this observation may be suggestive of different recombination activities for each locus taking place in *P. viridiflava* isolates (Figure S3). Strikingly, in all three cases the local celery isolates clustered distinctly and separately from the other local isolates. This observation contrasts with the view that *P. viridiflava* is not adapted to host plant species at the local level [16].

Even though the phytopathological and the biochemical profiling of the celery isolates were identical to the rest of the isolates examined, the differentiation seen in the molecular characterization led us to examine these isolates against a broader range of *P. viridiflava* strains by including *gyrB*, *rpoD* and *rpoB* sequences deposited in GenBank (Figures 3, 4, 5 and S3A). Our analysis revealed a very interesting pattern in which almost all the local isolates were grouped together and in separate clusters from the other isolates deposited in GenBank. This independent grouping of the Cretan isolates has been described previously for other plant pathogens [44,45]. The island of Crete, located in the south-central Mediterranean basin, constitutes an isolated terrestrial land part between three continents; Europe, Africa and Asia. Previous, reports suggested that plant pathogens within the bounds of Greek islands presented separated clades in the generated dendrograms, revealing a remarkable genome polymorphism compared to mainland Europe pathogen populations in which geographic correlations could not be established [44,45]. Thus, populations from different Greek islands were differentiated from each other, while genetic divergence was also found among subpopulations of the same plot. On the other hand, populations from mainland regions of Greece had high genotypic diversity. This indicates independent evolution of microorganisms in isolated geographic regions like Crete, as appears to be the case with the local *P. viridiflava* isolates.

However, in the *gyrB* phylogenetic tree, the tomato isolates as well as the blite and melon isolates did not group with the other local isolates groups (Figure 3) and the same was observed in the *rpoD* tree for the blite and melon isolates (Figure 4). This possibly indicates a recent arrival of these specific isolates. Unfortunately, we could not obtain consistent results from the *rpoB* tree due to insufficient number of deposited sequences in GenBank. These results indicate that the celery isolates may be adapted to host plant species at the local level.

Finally, our phylogenetic analysis supports the hypothesis that the intra-specific genetic variation of the *P. viridiflava* is not a result

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**Table 4. Local bacterial strains used in this study for gyrB, rpoD and rpoB phylogenetic analysis.**

| Strain No. | Host                  | *gyrB* GenBank No. | *rpoD* GenBank No. | *rpoB* GenBank No. | Origin                  |
|------------|-----------------------|--------------------|--------------------|--------------------|------------------------|
| PV271      | Apium graveolens L.   | JN383377           | JN383347           | JQ267553           | This study             |
| PV272      | Apium graveolens L.   | JN383378           | JN383348           | JQ267548           | This study             |
| PV272a     | Apium graveolens L.   | JN383379           | JN383349           | JQ267555           | This study             |
| PV273      | Apium graveolens L.   | JN383380           | JN383350           | JQ267550           | This study             |
| PV273a     | Apium graveolens L.   | JN383381           | JN383351           | JQ267552           | This study             |
| PV274      | Apium graveolens L.   | JN383382           | JN383352           | JQ267557           | This study             |
| PV276      | Apium graveolens L.   | JN383365           | JN383353           | JQ267556           | This study             |
| PV612      | Cucumis melo cv. Naudin | JN383366         | JN383354           | JQ267551           | This study             |
| PV527      | Amananthus blitum L.  | JN383367           | JN383355           | JQ267560           | This study             |
| PV3005     | Solanum melongena L.  | JN383368           | JN383356           | JQ267559           | This study             |
| PV3006     | Solanum melongena L.  | JN383369           | JN383357           | JQ267561           | This study             |
| PV570      | Acanthus mollis L.    | JN383370           | JN383358           | JQ267558           | This study             |
| PV574a     | Acanthus mollis L.    | JN383371           | JN383359           | JQ267554           | This study             |
| TK6615     | Solanum lycopersicum  | JN383372           | JN383360           | JQ267549           | This study             |
| PV441      | Solanum lycopersicum  | JN383373           | JN383361           | JQ267544           | This study             |
| PV442      | Solanum lycopersicum  | JN383374           | JN383362           | JQ267545           | This study             |
| PV608      | Cynara scolymus L.    | JN383375           | JN383363           | JQ267546           | This study             |
| PV609      | Cynara scolymus L.    | JN383376           | JN383364           | JQ267547           | This study             |

Information for other strains used, can be found in supplementary Table S3. doi:10.1371/journal.pone.0036090.t004
of host specific adaptation. However, an exception seen in our study was that of the celery isolates formed a distinct cluster separated from other \textit{P. viridiflava} strains and grouped apart from the other local isolates (Figures 3 and 4). This exemption needs to be further examined by including more geographically distant isolates in order to identify possible host- or geography-related genetic polymorphism. Moreover, the validation of the results against a broader range of samples, coupled with detailed phytopathological (e.g. determination of pathotypes, race resistance, etc.) and molecular attributes may provide a more relevant correlation among molecular and phytopathological traits at the intraspecific level in \textit{P. viridiflava}. This will be critical for obtaining a more comprehensive understanding of the biology, sources and shifts in genetic diversity and evolution of this species and should support the development of molecular identification tools and epidemiological studies in diseases caused by \textit{P. viridiflava}.

Materials and Methods

Isolation and identification of bacterial isolates

Affected plant parts, tissues or whole plants were collected and maintained in plastic bags at 6°C until isolations were performed. Samples from infected parts were surface disinfested by placing in 10% ethanol for 30 sec. After two thorough washings in sterile water, small pieces taken from the margin of the infected tissue
measured at 600 nm and by dilution plate counts. Control plants were sprayed with sterile distilled water.

Stem inoculations were made on tomato and chrysanthemum plants by stabbing with the tip of a sterile toothpick, previously dipped in individual colonies of each strain, into the plant stem just above the second true leaf. Controls were similarly treated with sterile toothpicks. All inoculated plants were held under greenhouse conditions (10–30°C) under intermittent mist (10 sec each hour). Symptoms were evaluated for one month after inoculation.

Cross-inoculation tests were made on detached capitulum bracts of artichoke, on snap bean (*Phaseolus vulgaris* L. cv. Kentucky Wonder) pods and on immature lemon fruits. Surface tissues were swabbed with 70% ethanol and washed in sterile distilled water and stabbed with a sterile needle at six sites. Inoculations were made by deposition of 15 μl of a bacterial suspension adjusted, as above, to approximately 10^6 cfu μl^-1. Ten artichoke bract leaves and two immature lemon fruit or bean pods were used for each strain. After inoculation, bracts, fruits and pods were kept in closed transparent plastic boxes lined with moist blotting paper, at room temperature (15–30°C). All inoculations sites were assessed daily for ten days to record disease symptoms.

**Bacterial cultures and genomic DNA preparation**

All *Pseudomonas* strains were grown at 26–28°C in King’s medium B broth for 24 h. From these cultures, cells were washed with sterile 10 mM MgCl_2_ and a cell suspension was prepared, which was adjusted to an OD_600_ of 0.4, corresponding to 200 cfu mL^-1. Aliquots of 500 μl in 2 mL cryo-tubes were stored at −80°C. For DNA extraction, the tube contents were thawed at room temperature, the cells were lysed for 1 h by addition of 100 μl of a bacterial suspension adjusted, as above, to approximately 10^6 cfu μl^-1. Ten artichoke bract leaves and two immature lemon fruit or bean pods were used for each strain. After inoculation, bracts, fruits and pods were kept in closed transparent plastic boxes lined with moist blotting paper, at room temperature (15–30°C). All inoculations sites were assessed daily for ten days to record disease symptoms.

**Molecular profiling**

Detailed characterization of the genetic variability among isolates belonging to *P. viridiflava* species was achieved by DNA fingerprinting, based on BOX-and ERIC-PCR (collectively known as rep-PCR) [23–25], as was previously described [52]. PCR reaction contained 150 ng template DNA, each of the deoxy nucleoside triphosphates at a concentration of 250 nM, primers at a total concentration of 2.5 μM, 2.5 mM MgCl_2_, and 2 units of Taq DNA polymerase (Kapa Biosystems) in a total volume of 20 μl. In the case of BOX-PCR the primer used was the BOXA1R (5’-
CTA CGG CAA GGC GAC GCT GAC G-3') while in the case of ERIC-PCR the primer pair was the ERIC1R/ERIC2 (5'-ATG TAA GCT CCT GGG GAT TCA C-3' and 5'-AGA TAA GTG ACT GGG GTG AGC G-3' respectively). The PCR reactions were performed in an Eppendorf Mastercycler Gradient according to the following program: 1 cycle at 95°C for 7 min, 30 cycles consisting of 1 min at 95°C, 30 sec at 53°C and 5 min at 72°C, and 1 cycle at 72°C for 15 min.

In both fingerprinting methods, the patterns were normalized and scorings were performed twice by two independent persons and the results obtained have no impact on the generated tree. The profiles of the rep-PCR gels were transformed into numerical data by P (band presence) and A (band absence) in order to be used for phylogenetic tree construction. Pairwise similarities between electrophoretic patterns were calculated with the Jaccard coefficient and clustering was carried out by the Unweighted Pair Group Method with Arithmetic mean (UPGMA), as previously described [34]. Phylogenetic analyses were conducted in MEGA (Molecular Evolutionary Genetics Analysis) version 5.0 software tool [53].

PCR amplification and sequencing of gyrB, rpoB and rpoD

PCR amplification of parts of the gyrB and rpoD genes was carried out following the method and primers described previously [1,54]. PCR reactions contained 150 ng of template DNA, each of the deoxynucleoside triphosphates at a concentration of 250 μM, total primers at a concentration of 2.5 μM, and 2 units of Taq DNA polymerase (Kapa Biosystems) in a total volume of 20 μl. PCR amplification was performed as follows: initial DNA denaturation at 94°C for 5 min, 35 cycles consisting of 1 min at 94°C, 1 min at 57°C and 2 min at 72°C, and a final step of 72°C for 10 min. Amplified products were electrophoresed on 1.5% agarose gels and purified using QIAquick columns (Qiagen) following the manufacturer's instructions. The nucleotide sequences of gyrB and rpoD genes were determined directly from the PCR fragments with the reading of the respective PCR amplicons in both directions, using the primer pair UP-E/APrU (5'- CAG GAA ACA GCT ATG ACC AYG SNG GNG GNA ART TYR-3' and 5'-TGT AAA ACG ACG GCC AGT GCN GGR TCY TTY TCY TGR CA-3' respectively) for gyrB gene and PvRpoD1/PvRpoD2 for rpoD gene (TGA AGG CGA RAT CGA AAT CGC CAA and 5'-YGC MGW CAG CTT YTG CTG GCA-3'). The sequences were further analysed with MEGA5 software [53].

Data analysis

Partial sequences of the three housekeeping genes, gyrB, rpoD and rpoB, were obtained from eighteen *P. viridiflava* strains (Table 1). Phylogenetic analysis was carried out using the partial sequences obtained plus corresponding sequences retrieved from NCBI GenBank. Sequence alignment was carried out using the program CLUSTALW [55] and corrected manually.

Phylogenetic trees were established using the UPGMA method [33] as in the dendrogram of Figure 2 or the Neighbour-Joining method [35] as in the dendrogram of Figures 3 and 4. The percentage of replicate trees in which the associated strains clustered together in the bootstrap test (1500 replicates; [56]) was estimated and is shown next to the tree branches. The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The evolutionary distances were computed using the Maximum Composite Likelihood method [57] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5.

As a measure of goodness of fit for cluster analysis the cophenetic correlation was used [58]. It derives from the comparison of the cophenetic value matrix against the matrix used for the generation of the clustering for 99 permutations. Firstly, the MEGA5 software was used for the estimation of pairwise Genetic Distances among all investigated genotypes. The generated pairwise matrix, regarded as the similarity matrix, was inserted into the SAHN module of NTSYSpc [50] for the generation of the UPGMA tree file and the COPH module of NTSYSpc for the generation of the cophenetic (ultrametric) value matrix. The 2 matrices were inserted into the MXCOMP module of NTSYSpc for the Mantel test. If r ≥ 0.9 the fit is interpreted as very good while an r value between 0.8 and 0.9 is interpreted as good fit.

Supporting Information

**Figure S1** *P. viridiflava* natural infections revealing leaf spots on eggplant seedlings (A), pith necrosis on tomato plants (B), leaf spots on celery (C) and bract leaves of artichoke (D).

**Figure S2** *P. viridiflava* isolates from different hosts did not produce deep black necrotic pit on detached immature lemon fruits (A), but caused rust-coloured lesions within 48 h on excised snap bean pods (B), had pectinolytic activity (C) and induced hypersensitive response on tobacco leaves (D).

**Figure S3** Phylogenetic trees of the local *P. viridiflava* isolates. The construction of the dendrogram was based on *A* gyrB gene sequence, *B* rpoD gene sequence and *C* rpoB gene sequence. The evolutionary history was inferred using the UPGMA method. The bootstrap consensus tree inferred from 1,500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA5.

**Table S1** Cross inoculation assays of *Pseudomonas* spp. and *Pseudomonas viridiflava* local isolates and reference strain. +: Compatible reaction. -: Incompatible reaction.

**Table S2** Comparison of *P. viridiflava* local isolates from different hosts found in the island of Crete and other fluorescent *Pseudomonas* species used in differential nutritional and biochemical tests. (+) = positive; (−) = negative; NT = not available.

**Table S3** Bacterial strains obtained from GenBank used for gyrB, rpoD and rpoB phylogenetic analysis.

Acknowledgments

The authors would like to thank Professor Nickolas J. Panopoulos for the critical reading of the manuscript. The authors would like to thank the two
anonymous referees for their careful English language and style corrections.

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Conceived and designed the experiments: PFS EAT DEG. Performed the experiments: PFS EAT EM. Analyzed the data: PFS EAT FNV DEG. Contributed reagents/materials/analysis tools: DEG FNV. Wrote the paper: PFS EAT.
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