Incidence, Speciation, and Morpho-Genetic Diversity of Penicillium spp. Causing Blue Mold of Stored Pome Fruits in Serbia

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Abstract: Blue mold, caused by Penicillium spp., is one of the most economically important postharvest diseases of pome fruits, globally. Pome fruits, in particular apple, is the most widely grown pome fruit in Serbia, and the distribution of Penicillium spp. responsible for postharvest decay is unknown. A two-year survey was conducted in 2014 and 2015, where four pome fruits (apple, pear, quince, and medlar) with blue mold symptoms were collected from 20 storage locations throughout Serbia. Detailed morphological characterization, analysis of virulence in three apple cultivars, and multilocus phylogeny revealed three main Penicillium spp. in order of abundance: P. expansum, P. crustosum, and P. solitum. Interestingly, P. expansum split into two distinct clades with strong statistical support that coincided with several morphological observations. Findings from this study are significant and showed previously undocumented diversity in blue mold fungi responsible for postharvest decay including the first finding of P. crustosum, and P. solitum as postharvest pathogens of quince and P. crustosum of medlar fruit in the world, and P. expansum of quince in Serbia. Data from this study provide timely information regarding phenotypic, morphological and genotypic plasticity in P. expansum that will impact the design of species-specific detection tools and guide the development of blue mold management strategies.

Keywords: postharvest decay; blue mold; Penicillium expansum; Penicillium crustosum; Penicillium solitum; pome fruit; fruit storage

1. Introduction

Pome fruits consist of apples (Malus domestica Borkh.), pears (Pyrus communis L.), quince (Cydonia oblonga Mill.), Asian pear (Pyrus seratina Rehd.), medlar (Mespilus germanica L.), and many other wild species of the Rosaceae family [1]. Most pome fruits are stored for extended periods of time (6 to 12 months) in a cold and controlled atmosphere. This allows fruit to be preserved and be of high quality so they can be available for year-round consumption and for trade to other countries. However, fruit rots reduce fresh fruit for consumption, negatively impact fruit quality, and contribute to mycotoxin contamination, specifically patulin, which is the case for Penicillium spp. [2].

Blue mold caused by Penicillium spp. is one of the most economically important postharvest diseases and a survey in Washington State revealed that it accounted for 28% of fruit decays in storage [3]. Blue mold is characterized by a soft, watery rot that is light brown in color accompanied by the appearance of blue-green conidia on the fruit surface that develops at advanced stages of decay. P. expansum Link., and other Penicillium spp. do not directly infect fruits, as they require wounds caused by stem punctures and bruises that occur before, during, and after harvest [2,4]. Conidia are the primary source of inoculum and are detectable in the packinghouse in flume water, on bin surfaces, fruit, and in the air [5–8]. Unfortunately, no resistance to blue mold is present in commercial apple cultivars as they are all susceptible [9].
Penicillium expansum is the most common and economically important causal agent of blue mold of stored apple and pear worldwide [9–11]. However, other species have been shown to be significant contributors of blue mold which include P. aurantiogriseum Dierckx, P. brevicompactum Dierckx, P. carneum Frisvad, P. chrysogenum Thom, P. commune Thom, P. crustosum Thom, P. dendriticum Pitt, P. digitatum (Pers.: Fr.) Sacc., P. glabrum (Wehmer) Westling, P. griseofulvum Dierckx, P. ramulosum Visagie & K. Jacobs, P. rugulosum Thom, P. solitum Westling and P. verrucosum Dierckx [5,6,12–15]. P. expansum has also been found to cause blue mold on quince [16]. However, it is well documented in the literature that P. expansum is the most common and aggressive species while P. solitum is a weak pathogen of apple and pears [6,17,18].

Apple is one of the most widely grown pomes in Serbia. In 2019, apples were cultivated on 26,089 ha, with a production of 499,578 tones, which comprises 3% of the total European apple production. Pear was produced on significantly less area, 4970 ha producing 54,859 t, representing 2.2% of the European pear market. Quince is a high value fruit in Serbia that is used to make jams, jellies, and brandy. Annual quince production is 11,074 t, from 1915 ha which provided 31% of total quince fruit grown in Europe. Serbia is the second largest quince producer in Europe and the ninth in the world [19]. P. expansum and P. crustosum have been described as causal agents of blue mold on apple [20–23] and pear fruit [24] in Serbia, and there are no data regarding incidence and distribution of blue mold fungal species on other pome fruits. Therefore, the aim of the current study was to identify the causal agents of blue mold on pome fruit in Serbia, gain insight into the diversity of Penicillium spp. via morphological and genetic characterization, and to evaluate the virulence of predominant species on different apple fruit cultivars.

2. Materials and Methods

2.1. Sample Collection and Fungal Isolation

In a two-year survey, during 2014 and 2015, four different pome fruits (apple, pear, quince and medlar) with blue mold symptoms were collected from 20 storage locations (Figure 1). Fungal isolation was done from the internal portion of decayed tissue from surface disinfected fruit in 70% ethanol, after aseptic removal of the skin. Fragments from the margin of healthy and decayed tissue were placed on potato dextrose agar (PDA, EMD, Darmstadt, Germany, pH 5.6 ± 0.2) in Petri dishes (90 mm). After 5 days incubation at 24 ± 2 °C in the dark, colony fragments were transferred to PDA to obtain pure cultures, which were used to obtain monosporial isolates. For long term maintenance, isolates were kept as conidial suspensions in 30% glycerol 0.05% agar 0.05% Tween 20 (Sigma-Aldrich, Burlington, MA, USA) at −80 °C.

2.2. Pathogenicity Test

‘Idared’ apple, ‘Williams’ pear, ‘Leskovačka’ quince and medlar (local cultivar) fruit obtained from a local market (IPM orchard origin), were washed and surfaced sanitized with 70% ethanol, then wounded with the point of a finishing nail (14 mm × 4 mm) on two opposite sides of the fruit at the equator. Spore suspensions (~10^6 spores/mL) were prepared by adding one fungal fragment (6 mm in diameter) from 7-day-old-cultures grown on PDA in 5 mL sterilized water with Tween 20 (0.05%) and adjusted using a haemocytometer [25,26]. Artificial inoculations were conducted by pipetting 40 µL of spore suspension into each wound and two fruit of every pome species were used per isolate. All isolates were inoculated onto apple fruit, whereas five isolates from pear were inoculated on pear, four isolates from quince onto quince and one from medlar onto medlar. Control fruit were wounded and inoculated with 40 µL of sterilized water containing Tween 20 (0.05%). Inoculated and control fruit were placed in plastic containers under high humidity and kept at 24 ± 2 °C for 7 days in natural light dark cycles. Re-isolation of the pathogen was performed as described above.
Re-isolation of the pathogen was performed as described above. under high humidity and kept at 24 ± 2 °C for 7 days in natural light dark cycles. (ribosomal internal transcribed spacer region (ITS, 600 bp), portions of the specific gene (α-tubulin, β-tubulin, ribosomal protein large subunit (RPB2, 1000 bp), nuclear ribosomal internal transcribed spacer region (ITS, 600 bp), portions of the β-tubulin (BenA, 511 bp), calmodulin (CaM, 580 bp) and RNA polymerase II second largest subunit (RPB2, 1000 bp) were amplified using ITS1/ITS4, Bt2a/Bt2b, CMD5/CMD6 and 5F/7CR primers, respectively [29–33]. PCR reaction mix (25 µL) contained 1 µL of template DNA, 1xPCR Master Mix (Thermo Scientific, Vilnius, Lithuania) and 0.4 µM of each primer. Conditions for Pepg1 amplification were: initial denaturation at 92 °C for 5 min, followed by 30 cycles at 92 °C for 1 min, 55 °C for 45 s and 72 °C for 45 s, and final elongation at 72 °C for 10 min. Conditions for amplification of ITS region were: initial denaturation at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s, and final elongation at 72 °C for 10 min. Conditions for BenA and CaM amplifications were: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 60 s, and final elongation at 72 °C for 7 min. Conditions for RPB2 amplification were: initial denaturation at 94 °C for 5 min, followed by 5 cycles at 94 °C for 45 s, 50 °C for 45 s and 72 °C for 60 s, then 5 cycles at 94 °C for 45 s, 52 °C for 45 s and 72 °C for 60 s and 30 cycles at 94 °C for 45 s, 55 °C for 45 s and 72 °C for 60 s. Final elongation occurred for 7 min at 72 °C [33]. PCR products (5 µL) were observed in 1.5% agarose gel, stained in ethidium bromide and visualized with UV transilluminator. Amplified products were purified and sequenced using forward and reverse primers to yield a 2X consensus
amplicon sequence. Sequences were assembled using Pregap4 from the Staden programme package [34] and deposited in the NCBI GenBank.

2.4. Multilocus Sequence Analysis and Phylogeny

The obtained fungal DNA sequences were compared with those publicly available using MegaBlast (http://www.ncbi.nlm.nih.gov/, accessed on 1 June 2021). Related sequences and those of the closest species were retrieved from GenBank and aligned with those obtained in this study using ClustalX [35], under MEGA version X [36]. Evolutionary history was inferred based on individual and combined analyses of four loci (ITS, BenA, CaM, and RPB2) of 26 isolates obtained in this study, reference isolates and *Penicillium lanosoceruleum* CBS 215.30 as an outgroup (Table 1), using the Maximum Likelihood (ML) and Maximum Parsimony (MP) methods (MEGA X). For ML, the best nucleotide substitution model was determined using the “find best model” option in MEGA X. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. The MP trees were obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 3, in which the initial trees were obtained by the random addition of sequences (ten replicates). To estimate the statistical significance of the inferred clades, 1000 bootstraps were performed.

2.5. Cultural Morphology

For 26 selected isolates, colony morphology (appearance, presence of exudate, reverse color) and growth were analysed on Malt Extract Agar (MEA), Czapek Yeast Extract Agar (CYA), and Yeast Extract Sucrose Agar (YES) as described by Visagie et al. [33], and additionally on Potato Dextrose Agar (PDA). Plates were inoculated at three points with 1 µL of spore suspension (10^6 conidia/mL, as stated for pathogenicity test) of each isolate. Three plates were used per media. All inoculated plates were kept in the dark at 24 ± 2°C. Morphology of conidiophores and conidia, type of conidiophores and their ornamentation were evaluated from 7–10 day-old-cultures grown on MEA at 24 ± 2°C [18,33] using compound microscope Zeiss Axio Lab, Jena, Germany. Photographs of conidiophores and conidia and their sizes were obtained using camera: Axiocam ERc 5s, Zeiss and software ZEN 2 (blue edition), Jena, Germany.

2.6. Ehrlich Test

The production of cyclopiazonic acid and other alkaloids was examined according to Lund [37]. Filter paper (20 mm × 20 mm) was immersed in Ehrlich reagent (2 g 4-dimethylamino-benzaldehyde in 85 mL 96% ethanol, with 15 mL 10 N HCl) and was placed on top of the mycelial side of agar plugs (three plugs per isolate, diameter 8 mm) from 7 day-old-cultures grown on CYA at 24 ± 2°C. After 2–10 min incubation, color or color change were recorded.

2.7. Virulence Assessment in Apple Fruit

Fungal virulence was assessed using 15 selected isolates of *Penicillium* spp. (seven *P. expansum*, seven *P. crustosum* and one *P. solitum* isolate) (Table 1) on three apple cultivars: ‘Golden Delicious’, ‘Red Delicious’, and ‘Granny Smith’. Mature apple fruit were wound-inoculated as described in the pathogenicity test. Each apple fruit was inoculated with 40 µL of spore suspension on two opposite sides of the fruit. Spore suspensions were adjusted to concentration 10^5 conidia/mL using a haemocytometer [25,26]. Two apple fruit were used per cultivar (six per isolate). Control fruit were wounded and inoculated containing 40 µL of sterilized water with Tween 20 (0.05%). All fruit were kept under moist conditions in sterile plastic boxes at 24 ± 2°C temperature under natural light dark cycles. Lesion size was measured at the equatorial and longitudinal axes at 7- and 9-days post inoculation (dpi).
2.8. Statistical Analysis

Kolmogorov–Smirnov and Shapiro–Wilk tests were used to check for normal distribution of the data and Levene test assessed homogeneity of variances. Some of the data were not normally distributed, and homogeneity of variances was violated. Due to large sample sizes parametric tests were used [38,39]. To avoid type I error, we chose more stringent conditions by using $p = 0.01$ as a significance level. Two-way ANOVA was used to determine which species and culture media affected colony size, and how species and cultivar impacted lesion diameter. One-way ANOVA was used to determine if there was a difference in colony size between isolates, species or between culture media; difference in sizes of conidiophore parts (ramus, metulae, phialide and conidia) between isolates or species and to determine a difference in lesion size between isolates, species or cultivars. Post hoc Tukey HSD test was used to evaluate differences that occurred in testing multiple groups (between isolates of same species or between three species). Evaluating effect size of two factors on the dependent variable was measured using partial eta squared ($\eta^2$). $\eta^2 < 0.06$ is considered as weak; $\eta^2 > 0.06$ moderate and $\eta^2 > 0.14$ as strong effect [40]. The statistical analyses were conducted with IBM SPSS 22 software (SPSS Inc., Chicago, IL, USA).

2.9. Cluster Analysis

To interpret the similarities and differences amongst *P. expansum* or *P. crustosum* isolates, a multivariate statistical analysis with hierarchical cluster analysis based on Ward’s method and Euclidian distance interval was used. To prevent dominance by large values at the expense of small ones, data were z-standardized using the formula: $z = (x - \mu)/\sigma - 1$. IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA), was used for statistical analysis.

2.10. Principle Component Analysis

To enable a comprehensive assessment of data obtained in this study, a Principal Component Analysis (PCA) with PAST 3.17 software [41] was used. To prevent dominance in the PCA by large values at the expense of small ones, data were z-standardized using the formula: $z = (x - \mu)/\sigma - 1$. Morphological characteristics (colony diameter on all tested media, conidia, metulae, and rami width, and metulae length, presence of yellow pigment in PDA and MEA reverse, and CYA reverse color) were analysed for 26 isolates, while both morphology and virulence were assessed for 14 select isolates.

### Table 1. Isolates used in this study.

| Isolate | Geographic Origin/Host cv. | Date     | GenBank Accession Number | References |
|---------|---------------------------|----------|--------------------------|------------|
|         |                           |          | ITS                     | BenA       | CaM       | RPB2      |            |
| JRad4   | Serbia, Radmilovac/Apple ‘Gloster’ | December 2014 | MZ364021   | MZ364047   | MZ364097   | MZ364075   | This study |
| 3JC6    | Serbia, Čelarevo/Apple ‘Braeburn’  | January 2015 | MZ364023   | MZ364056   | MZ364098   | MZ364076   | This study |
| 3JC11   | Serbia, Čelarevo/Apple ‘Granny Smith’ | April 2015 | OK432548    | MZ364048   | MZ364099   | MZ364077   | This study |
| 3JC23   | Serbia, Čelarevo/Apple ‘Modi’   | April 2015 | MZ364024   | MZ364049   | MZ364100   | MZ364078   | This study |
| 3JB13   | Serbia, Brestovik/Apple ‘Jonagold’ | January 2015 | MZ364025   | MZ364050   | MZ364101   | MZ364079   | This study |
| 3JB22   | Serbia, Brestovik/Apple ‘G. Delicious’ | January 2015 | MZ364026   | MZ364061   | MZ364102   | MZ364080   | This study |
| 3SD3    | Serbia, Smederevo/Apple ‘R. Delicious’ | January 2015 | MZ364027   | MZ364051   | MZ364103   | MZ364081   | This study |
| 3SD5    | Serbia, Smederevo/Apple ‘R. Delicious’ | January 2015 | MZ364028   | MZ364052   | MZ364110   | MZ364082   | This study |
| S1      | Serbia, Šid/Apple ‘R. Delicious’   | January 2015 | MZ364029   | MZ364037   | MZ364104   | MZ364083   | This study |
| 3MR1    | Serbia, Mala Remeta/Apple ‘G. Delicious’ | January 2015 | MZ364030   | MZ364035   | MZ364105   | MZ364084   | This study |
| KSA5    | Serbia, Šabac/Pear ‘Passe Crassane’ | March 2015 | MZ364031   | MZ364045   | MZ364111   | MZ364071   | This study |
| JMR2o * | Serbia, Mala Remeta/Apple ‘Fuji’    | November 2015 | MZ364033   | MZ364058   | MZ364108   | MZ364085   | This study |
**Table 1. Cont.**

| Isolate       | Geographic Origin/Host cv.                  | Date         | GenBank Accession Number | References            |
|---------------|-------------------------------------------|--------------|--------------------------|-----------------------|
|               |                                           |              | ITS                      | References            |
| JMR22 *       | Serbia, Mala Remeta/Apple 'Fuji'           | November 2015| MZ364034 MZ364059 MZ364109 MZ364086 | This study            |
| JBA8b *       | Serbia, Bavanište/Apple 'Jonagored'        | December 2015| MZ364035 MZ364054 MZ364106 MZ364087 | This study            |
| JPN2 *        | Serbia, Paraćin/Apple 'Idared'             | December 2015| MZ364036 MZ364055 MZ364107 MZ364088 | This study            |
| KPN4 *        | Serbia, Paraćin/Pear 'Poire de Cure'       | December 2015| MZ364032 MZ364046 MZ364112 MZ364072 | This study            |
| DBA5 *        | Serbia, Bavanište/Quince 'Leskovačka'      | December 2015| MZ364022 MZ364062 MZ364114 MZ364073 | This study            |
| DRH4a *       | Serbia, Ritepek/Quince 'Leskovačka'        | December 2015| MZ364020 MZ364060 MZ364113 MZ364074 | This study            |
| Penicillium crustosum |                           |              |                          |                       |
| JBA8a *       | Serbia, Bavanište/Apple 'Jonagored'        | December 2015| MZ364037 MZ364067 MZ389067 MZ364089 | This study            |
| JBA11 *       | Serbia, Bavanište/Apple 'Šifra'            | December 2015| MZ364038 MZ364068 MZ389061 MZ364090 | This study            |
| KGR2 *        | Serbia, Grocka/Pear 'Santa Maria'         | October 2015  | MZ364039 MZ364063 MZ389062 MZ364091 | This study            |
| KRI1P *       | Serbia, Ritepek/Pear 'Williams'           | December 2015| MZ364040 MZ364064 MZ389063 MZ364092 | This study            |
| KVA8 *        | Serbia, Valjevo/Pear 'Poire de Cure'       | December 2015| MZ364041 MZ364065 MZ389064 MZ364093 | This study            |
| DRH4b *       | Serbia, Ritepek/Quince 'Leskovačka'        | December 2015| MZ364042 MZ364066 MZ389065 MZ364094 | This study            |
| MHI4 *        | Serbia, Ritepek/Medlar local cv.           | December 2015| MZ364043 MZ364069 MZ389066 MZ364095 | This study            |
| Penicillium solitum |                           |              |                          |                       |
| DRI3 *        | Serbia, Ritepek/Quince 'Leskovačka'        | December 2015| MZ364044 MZ364070 MZ364115 MZ364096 | This study            |
| NCBI isolate  |                                           |              |                          |                       |
| Penicillium expansum |                           |              |                          |                       |
| CBS 325.48 ** | USA/Apple fruit                           | 2014         | MG714838 MG714864 MG714821 MG714845 | [44]                 |
| F758          | USA, Idaho/ Sugar beet                     | 2014         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| PCAS          | Italy/Chestnut                             | 2015         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| P34           | Italy/Withered grape                       | 2017         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| 4             | Greece/Kiwi fruit                          | 2017         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| CV 2860       | South Africa/Fynbos biome                 | 2015         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| CV 2863       | South Africa/Fynbos biome                 | 2015         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| LUB           | Serbia, Ub/Onion                           | 2015         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| Penicillium marneum |                           |              |                          |                       |
| CBS 109550 ** | Japan/Sandy soil                           | 2014         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| CBS 109547    | Tunisia/Sandy soil                         | 2015         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| Penicillium crustosum |                           |              |                          |                       |
| CBS 115503 ** | Scotland, Aberdeen/Lemon                   | 2015         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| CV 0241       | South Africa/Fynbos biome                 | 2015         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| 5A            | Italy/Chestnut                             | 2015         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| N2AS          | Serbia/Nectarine                           | 2021         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| Penicillium echinulatum |                           |              |                          |                       |
| CBS 317.48 ** | Canada, Culture contaminant                | 2015         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
| DTO22884      | Unknown/Unknown                            | 2015         | MG821365 MG821365 MG821365 MG821365 | [45]                 |
Table 1. Cont.

| Isolate     | Geographic Origin/Host cv. | Date   | GenBank Accession Number | References |
|-------------|---------------------------|--------|--------------------------|------------|
|             |                           |        | ITS                      | BenA       | CaM         | RPB2       |            |
| Penicillium solitum |                           |        |                         |            |             |            |            |
| CBS 424.89 ** | Germany/Unknown           |        | AY3739321               | AY6743542  | KU8968511   | KU9043631  | 1[42]; 2[50] |
| XF          | Italy/Chestnut            | 2015   | MG821373                | MF100861   | MF100881    | /          | [45]       |
| Penicillium discolor |                       |        |                         |            |             |            |            |
| CBS 474.84 ** | Israel/Raphanus sativus   |        | AJ004816                | AY674348   | KU896834    | KU904351   | [42]       |
| DTO047A2    | Unknown/Unknown           |        | /                       | MN149922   | MN149941    | MN149961   | [52]       |
| Penicillium lanosocoeruleum |                  |        |                         |            |             |            |            |
| CBS 215.30  | USA/Culture contaminant   |        | NR_1635411              | KU8968172  | JX9966721   | JX9967121  | 1[51]; 2[42]; 3[54] |

* Isolates used in virulence study; ** Ex-type/referent isolates.

3. Results

3.1. Blue Mold Symptoms and Koch’s Postulates

Pome fruits, sampled from 20 different locations in Serbia, exhibited typical blue mold symptoms. The decayed area was light brown, soft and watery, and easily separated from the healthy tissue, and ranged from small lesions to completely decayed fruit. In most cases decayed fruit were covered with blue-green colored spores, and had an earthy, musty odour. In total, 96 Penicillium spp. isolates were obtained, 71 originating from apples, 14 from pears, 10 from quince, and one from medlar. All isolates were pathogenic and caused decay on inoculated healthy ‘Idared’ apple fruit, whereas five isolates from pear were pathogenic to pear, four isolates from quince to quince and one from medlar to medlar. P. crustosum induced slightly darker colored decay on inoculated pome fruits than P. expansum, which was the most evident on fruits with yellow skin. Control fruits remained symptomless. On inoculated fruits, blue-green sporulation was mainly present at the inoculation site. Reisolated fungi exhibited identical morphological characteristics that mirrored the original isolate, thus completing Koch’s postulates. Blue mold on representative inoculated pome fruits is presented in Figure 2.

3.2. Molecular Identification and Single Nucleotide Polymorphism

P. expansum specific primers (PEF/PER) generated amplicons of 404 bp in 88 out of 96 tested isolates, while no amplification was observed in 8 isolates and the negative controls. For ITS, BenA, CaM, and RPB2 amplicons of expected size (600, 511, 580, 1000 bp, respectively) were obtained in 26 selected isolates (18 isolates which were positive with P. expansum specific primers and 8 isolates other than P. expansum). Sequencing of the obtained amplicons yielded nucleotide sequences 559–561 nt long for ITS, 428–432 nt for BenA, 501–504 nt for CaM, and 971 nt for RPB2, excluding primers, which were deposited in the NCBI GenBank under accession numbers given in Table 1.

The identity of isolates was confirmed based on single and multilocus phylogeny. Therefore, 18 isolates were identified as P. expansum and confirmed the results of specific primer identification, while seven isolates were identified as P. crustosum, and one as P. solitum. Molecular identification (total of 96 Penicillium isolates) revealed that 91.64% of blue mold on pome fruit collected in this study was caused by P. expansum, 7.29% P. crustosum and 1.04% P. solitum.
Multiple sequence alignment of the *P. expansum* isolates revealed that: (i) no sequence variation was identified in ITS amplicons, (ii) two sequence variants were found in *BenA* sequences (2 nt differences) and *CaM* (3 nt differences), and (iii) three sequence variants were identified in *RPB2* (1, 14 and 15 nt differences) (Table 2). *Penicillium expansum* ITS sequences were identical to those from *P. expansum* deposited in NCBI GenBank (i.e., AY373912, MG714838, and MG821365). One *BenA* variant (11 isolates) had sequences that matched several *P. expansum* sequences (i.e., JX091539, JX091540) and the other *BenA* variant (7 isolates) had sequences in line with *P. expansum* (i.e., MH040784) deposited in NCBI. One *CaM* variant (11 isolates) was identical with *P. expansum* sequences deposited in NCBI GenBank (e.g., DQ911134, MG714821). The other *CaM* variant (7 isolates) differed in 3 nt. One *RPB2* variant (12 isolates) was identical with *P. expansum* sequences JF417427 and MG714845, while the second *RPB2* variant (four isolates) showed 14 nt differences, and the third (two isolates) had 1 nt difference. Between second and third *RPB2* variants, the difference was in 15 nt (Table 2).

Multiple sequence alignment of the *P. crustosum* isolates revealed that: (i) no sequence variation was identified in sequences of ITS and *BenA*, (ii) two sequence variants occurred in *CaM* genomic fragments (1 nt differences), and (iii) four variants were identified in *RPB2* (having 1, 1, 1, 2 nt differences) (Table 2). ITS and *BenA* from *P. crustosum* sequences (seven isolates) were identical with those from *P. crustosum* isolate CV 0241, i.e., JX091403 and JX091536, respectively. Based on the *CaM* locus, six isolates were identical, and matched sequences of the *P. crustosum* isolate CV 0241 (JX141576) and CBS 115503 (DQ911132), while the sequence of one isolate, JBA8a differed from the others in 1 nt. One *RPB2* variant (two isolates) was identical with the sequence from *P. crustosum* isolate CBS 115503 (MN969914), while the second *RPB2* variant (two isolates) matched CV 0241 (MNI149972), and the variants differed in 2 nt. The third *RPB2* variant (two isolates) differed in 2 nt from both
the first and the second variant. The fourth variant (one isolate) differed in 3, 3, and 1 nt from the first, second, and third variant, respectively.

Table 2. Haplotype analysis of Penicillium expansum and P. crustosum isolates.

| Sequence | Position |
|----------|----------|
| **Penicillium expansum** |          |
| **BenA** | 232 260  |
| I variant | T C |
| II variant | G T |
| **CaM** | 79 154 356 |
| I variant | T C A |
| II variant | C T G |
| **RPB2** | 139 151 325 340 373 454 520 760 841 854 889 943 958 979 1036 |
| I variant | C A G T G C T C T G A C C |
| II variant | T G A C A T C T C T C |
| III variant | C A G T G C T C C A C T T |
| IV variant | C A G T G C T C C |
| **Penicillium crustosum** |          |
| **CaM** | 303 337 414 |
| I variant | C A T |
| II variant | - G - |
| **RPB2** | 175 724 940 979 |
| I variant | T C A C |
| II variant | T C C T |
| III variant | T C C C |
| IV variant | C T - |

Gray background represents differences amongst variants.

P. solitum obtained ITS and CaM sequences were identical with those of P. solitum CBS 424.89 (AY373932 and KU896851, respectively) and BenA had the highest similarity with CBS 424.89 (AY674354), while RPB2 aligned with P. solitum CBS 424.84 (KU904363).

3.3. Macromorphology

*P. expansum* isolates formed white mycelia (visible as white margins) with blue green conidia on all media. On PDA, colonies were mostly fasciculate with or without concentric zones and radially sulcate. Colony margins were entire. Exudate was absent. Colony reverse in most isolates was cream to pale yellow except for isolates 3JC6, 3S1 (intense yellow with brown ring around centre), KSA5 (intense yellow with cream centre), and JMR2z (dark yellow). On MEA, *P. expansum* formed fasciculate colonies mostly with concentric zones and were radially sulcate. Scarce clear exudate droplets were present on the colony edge. Colony reverse was in most isolates cream coloured, while in some yellow (3JC6, KSA5, and JMR2z—intense yellow with cream centre; 3S1—yellow with light brown ring). On CYA, colonies were concentrically fasciculate and weakly radially sulcate, rarely velutinous. Colonies were dense and differed in exudate production from no exudate, scarce, to abundant exudate droplets. Colony reverse varied from cream, yellow, salmon pink to red. Most isolates formed salmon pink to red reverse of different intensities, some formed yellow reverse (3JC6, 3S1, and KSA5) and some cream reverse (3JB22, JMR2o, and DBA5). On YES, colonies were mostly fasciculate with or without concentric zones, radially sulcate, sometimes velutinous (3JC23, KPN4, and DRI4a). Conidia color was blue green to grey, and colonies were sometimes cream around the centre, and exudate was absent. Colony reverse was pale yellow to intense yellow, with or without orange centre (Figure 3a,b).
P. crustosum isolates formed white mycelia (visible as white margins) with blue dull green conidia on all media. Colonies on PDA and MEA were similar: velutinous, sometimes radially sulcate, which become crustose after 10 days. Colony margins were white and exudate was absent. Colony reverse was cream to intense yellow. On CYA, P. crustosum formed velutinous, sometimes radially sulcate colonies mainly with irregular margins. Colonies were with or without exudate. Colony reverse was cream to yellow. On YES, colonies were velutinous, radially sulcate and dense. Conidia color was blue green and sometimes grey. Margins were entire and exudate was absent. Colony reverse was yellow to intense yellow (Figure 3a,b). Among P. crustosum one isolate (DRI4b) differed by forming concentrically floccose to fasciculate colonies on CYA and YES, and orange reverse on PDA, MEA, and CYA.

P. solitum isolate formed white mycelia with blue green conidia on all media. On PDA, colony was velutinous to floccose with concentric zones, without exudate. Colony reverse was orange with a cream margin. On MEA, the colony was floccose and radially sulcate, without exudate. Colony reverse was cream to light brown at the margin. On CYA, colony was floccose and radially sulcate, and abundant clear exudate droplets were present. Colony reverse was cream with a bright orange centre. On YES, colony was floccose and radially sulcate with bright blue grey conidia. Exudate was absent. Colony reverse was intense yellow (Figure 3a,b).

3.4. Colony Growth

Colony growth of P. expansum, P. crustosum and P. solitum on four media is presented in Table 3. The most favourable growth media for all three species was YES, followed by CYA and PDA, while colony diameter was smaller on MEA ($p < 0.01$). P. expansum growth was faster on CYA and YES compared to P. crustosum ($p < 0.0001$) (and P. solitum ($p < 0.0001$)), while on PDA and MEA was the same as P. crustosum ($p = 0.901$). P. solitum had the slowest colony growth on all media ($p < 0.01$). Variability in colony growth was observed within isolates of P. expansum (on all media) ($p < 0.01$) and P. crustosum (on MEA, CYA, and YES) ($p < 0.01$). Among P. expansum two groups of isolates were observed, fast growing (11 out of 18) and slower growing (7 out of 18) (Table 3, Figure 4). Two-way ANOVA revealed that both species and culture media had a large influence on colony growth. Variation in colony growth of 20.9% ($\eta^2 = 0.209, p < 0.0001$) is due to differences between species, while 17.3% ($\eta^2 = 0.173, p < 0.0001$) is caused by differences in culture media. Combined effect of species characteristics and culture media on its growth was negligible 2.6% ($\eta^2 = 0.026, p < 0.0001$). We did not observe that isolate origin had an influence on colony growth (data not shown).
Table 3. Colony diameter of *Penicillium, expansum*, *P. crustosum* and *P. solitum* isolates on different media 7 dpi, in the dark at 24 ± 2 °C.

| Isolate  | Colony Diameter on Different Culture Media (mm ± Standard Deviation) | PDA       | MEA          | CYA          | YES          |
|----------|--------------------------------------------------------------------|-----------|--------------|--------------|--------------|
|          |                                                                    | 37.22 ± 0.73hi | 48.72 ± 1.25b | 51.31 ± 1.21d-f |
| JRad4    | 47.00 ± 0.97f *                                                   | 30.67 ± 1.08b-d | 44.18 ± 1.59d-g | 49.71 ± 1.78c-f |
| 3JC11    | 42.69 ± 0.86d-f                                                  | 31.58 ± 0.69c-e | 43.09 ± 1.10c-f | 52.44 ± 0.63ef |
| 3JC23    | 45.81 ± 1.02ef                                                   | 29.42 ± 1.48a-c | 46.13 ± 1.09e-h | 50.53 ± 0.85d-f |
| 3JB13    | 40.58 ± 0.81b-e                                                 | 31.06 ± 0.97c-e | 46.94 ± 1.57f-h | 51.69 ± 1.14d-f |
| 3SD3     | 43.85 ± 1.26d-f                                                 | 30.72 ± 0.89b-d | 46.34 ± 1.66e-h | 53.28 ± 1.17f  |
| 3SD5     | 43.92 ± 0.97d-f                                                 | 32.08 ± 1.03de | 47.73 ± 1.02gh | 49.10 ± 1.54b-f  |
| 3MR1     | 44.83 ± 1.20d-f                                                 | 36.69 ± 2.12gh | 44.39 ± 6.26e-h | 49.64 ± 9.88c-f  |
| JBA8a    | 43.47 ± 7.05d-f                                                 | 37.89 ± 1.92hi | 42.42 ± 5.53b-e | 48.39 ± 8.64b-f  |
| JPN2     | 43.92 ± 7.85d-f                                                 | 39.03 ± 3.58i | 44.50 ± 5.96e-h | 48.89 ± 8.77b-f  |
| KPN4     | 44.56 ± 8.03d-f                                                 | 38.36 ± 1.50gh | 42.47 ± 0.63b-e | 48.03 ± 1.47b-f  |
| DRI4a    | 44.91 ± 7.60d-f                                                 | 37.50 ± 2.81i | 45.14 ± 6.31e-h | 50.21 ± 8.46d-f  |
| 3JC6     | 36.77 ± 0.87a-c                                                 | 27.33 ± 0.49a | 46.22 ± 0.86e-h | 49.31 ± 1.01b-f  |
| 3JB22    | 39.33 ± 1.42b-d                                                 | 28.64 ± 1.41ab | 38.69 ± 0.67a-c | 45.00 ± 2.61b-d  |
| 3S1      | 32.92 ± 1.62a                                                   | 36.38 ± 1.50gh | 35.53 ± 1.16a  | 41.44 ± 4.35ab  |
| KSA5     | 36.64 ± 0.70ab                                                 | 27.25 ± 0.46a | 38.36 ± 1.12ab | 42.50 ± 1.72ab  |
| JMR2o    | 35.03 ± 4.24ab                                                 | 32.06 ± 1.03de | 38.39 ± 3.21ab | 37.53 ± 2.17a   |
| JMR2z    | 42.41 ± 6.47c-f                                                 | 34.75 ± 2.06fg | 39.94 ± 2.43a-d | 45.72 ± 6.56b-e  |
| DBA5     | 36.78 ± 4.56a-c                                                | 33.17 ± 1.20ef | 36.28 ± 2.15a | 43.00 ± 5.54a-c  |
| Avg.     | 41.45 ± 5.76b **                                               | 32.94 ± 4.00b | 43.17 ± 4.75c | 48.03 ± 6.25c   |

*Penicillium crustosum*

| Isolate  | Colony Diameter on Different Culture Media (mm ± Standard Deviation) | PDA       | MEA          | CYA          | YES          |
|----------|--------------------------------------------------------------------|-----------|--------------|--------------|--------------|
| JBA8a    | 39.58 ± 5.18a                                                    | 30.39 ± 1.12a | 36.00 ± 2.54a | 41.61 ± 5.13ab |
| JBA11    | 36.38 ± 3.09a                                                   | 32.39 ± 1.36ab | 39.13 ± 6.09ab | 44.09 ± 6.19ab |
| KGR2     | 38.36 ± 2.84a                                                   | 31.34 ± 1.57ab | 38.69 ± 3.28ab | 40.92 ± 4.72a |
| KRI1P    | 37.91 ± 3.37a                                                   | 31.64 ± 1.60ab | 38.92 ± 3.79ab | 41.58 ± 5.12ab |
| KVA8     | 37.71 ± 3.07a                                                   | 33.25 ± 1.40bc | 35.53 ± 1.69a | 41.44 ± 4.35ab |
Table 3. Cont.

| Isolate | Colony Diameter on Different Culture Media (mm ± Standard Deviation) |
|---------|---------------------------------------------------------------|
|         | PDA              | MEA              | CYA              | YES              |
| MRI4    | 39.71 ± 4.10a    | 35.26 ± 2.48c    | 40.53 ± 5.64ab  | 48.47 ± 8.46b    |
| Avg.    | 38.39 ± 3.64b    | 32.77 ± 2.45b    | 38.90 ± 5.05b   | 43.51 ± 6.67b    |

*Penicillium solitum*

| DRI3    | 28.02 ± 0.50a    | 24.31 ± 0.68a    | 29.75 ± 0.69a   | 34.53 ± 1.22a    |

*Numbers with different letters represent a statistically significant difference in colony diameter within isolates of the same species per media according to Tukey HSD test (p < 0.01). ** Bolded numbers with different letters represent significant difference in colony diameter among species per media according to Tukey HSD test (p < 0.01).*

Figure 4. Dendogram showing the grouping of *Penicillium expansum* isolates based on colony diameter on different media (PDA, MEA, CYA, and YES), reverse on PDA and MEA (cream or yellow), and CYA reverse characteristics, using Ward’s model with Euclidian Distance interval. *P. expansum* isolates within a clade are indicated in black boxes.

3.5. Micromorphology

*P. expansum* formed mostly terverticillate and sometimes biverticillate conidiophores with smooth walls. Conidia were grey green to blue green in color, smooth, globose, subglobose, and elliptical, with average conidia size $3.24 \pm 0.30 \times 2.73 \pm 0.24 \mu m$. Phialides were smooth, mostly cylindrical and sometimes ampuliform, with average size $10.14 \pm 1.32 \times 2.94 \pm 0.47 \mu m$. Metulae ($12.94 \pm 2.30 \times 3.61 \pm 0.65 \mu m$) and rami ($19.66 \pm 4.47 \times 3.96 \pm 0.97 \mu m$) were smooth and cylindrical. *P. crustosum* formed mostly terverticillate and sometimes biverticillate conidiophores with evidently roughened walls. Conidia were dull green to blue green, smooth, globose to subglobose, with average conidia size $3.33 \pm 0.27 \times 3.06 \pm 0.23 \mu m$. Phialides were smooth, rarely roughened, ampuliform to cylindrical, and its average size was $10.05 \pm 1.35 \times 3.10 \pm 0.41 \mu m$. Metulae were roughened to smooth and cylindrical ($14.02 \pm 2.43 \times 3.86 \pm 0.54 \mu m$), while rami were
roughened and cylindrical (21.23 ± 4.45 × 4.11 ± 0.60 μm). Stipes were also roughened. *P. solitum* formed mostly terverticillate and sometimes biverticillate conidiophores with smooth or finely roughened walls. Conidia were smooth, mostly globose sometimes sub-globose, with average size 3.67 ± 0.17 × 3.42 ± 0.18 μm. Color of conidia was grey green to blue green, the same as *P. expansum* conidia. Phialides were smooth, ampuliform to cylindrical, with average size 10.13 ± 1.92 × 3.11 ± 0.41 μm. Metulae were smooth and cylindrical (12.20 ± 2.03 × 4.21 ± 0.56 μm), while rami were smooth to finely roughened and cylindrical (18.17 ± 4.09 × 4.41 ± 0.69 μm). Stipes were roughened. Comparative micromorphology (Table 4) between *P. expansum* and *P. crustosum* revealed: (i) there was no difference in conidia length (*p* = 0.09), while conidia of *P. expansum* were smaller in width than *P. crustosum* (*p* < 0.0001) (*P. solitum* isolate had significantly larger conidia than *P. expansum* and *P. crustosum* (*p* < 0.0001); (ii) there was no difference in phialides length (*p* = 0.75), and width (*p* = 0.011); (iii) metulae length and width were larger in *P. crustosum* compared to *P. expansum* (*p* < 0.0001) (*P. solitum* formed the widest metulae); (iv) rami were of similar size (*p* = 0.368 for length, *p* = 0.177 for width) (*P. solitum* formed the shortest and widest rami); (v) difference between investigated species was large in conidia width (*η^2^ = 0.175), moderate in conidia length (*η^2^ = 0.061), small in metulae and rami length and width (*η^2^ = 0.047; *η^2^ = 0.055 for metulae and *η^2^ = 0.03; *η^2^ = 0.023 for rami), (vi) variability within *P. expansum* isolates was observed in conidia, phialide, metulae, and rami size, while within *P. crustosum* isolates in conidia size and phialide length (*p* < 0.01). Isolate origin did not impact conidia or conidiophore size (statistical data not shown).

**Figure 4.** Dendogram showing the grouping of *Penicillium expansum* isolates based on colony diameter on different media (PDA, MEA, CYA, and YES), reverse on PDA and MEA (cream or yellow), and CYA reverse characteristics, using Ward’s model with Euclidian Distance interval. *P. expansum* isolates within a clade are indicated in black boxes.

**Figure 5.** Dendogram showing grouping of *Penicillium crustosum* isolates based on colony diameter on different media (PDA, MEA, CYA, and YES), using Ward’s model with Euclidian Distance interval. *P. crustosum* isolates within a clade are indicated in black boxes.
Table 4. Micromorphology of *Penicillium expansum*, *P. crustosum* and *P. solitum* isolates.

| Isolate            | Average Size (μm ± Standard Deviation) |
|--------------------|----------------------------------------|
|                    | Length (μm) | Conidia | Width (μm) | Phialides | Width (μm) | Metulae | Width (μm) | Rami | Width (μm) |
| *Penicillium expansum* |            |        |            |           |            |         |           |      |            |
| JRA4               | 3.25 ± 0.25c-e | 2.72 ± 0.19bc | 9.74 ± 1.38ab | 2.84 ± 0.55a-d | 12.72 ± 2.41a-e | 3.35 ± 0.59a-e | 19.64 ± 4.20a-c | 3.67 ± 0.63ab |
| JQC11              | 3.13 ± 0.30a-d | 2.56 ± 0.26ab | 10.06 ± 0.91a-d | 2.85 ± 0.34a-d | 11.63 ± 1.46a | 3.19 ± 0.38ab | 18.61 ± 2.10a-c | 3.58 ± 0.43a |
| JQC23              | 3.13 ± 0.24a-d | 2.52 ± 0.18a | 9.09 ± 1.35a | 2.89 ± 0.53a-e | 11.99 ± 2.65a-c | 3.23 ± 0.68a-c | 16.67 ± 3.51a | 3.78 ± 0.77ab |
| JQB33              | 3.00 ± 0.22ab | 2.52 ± 0.19a | 9.82 ± 0.77ab | 2.69 ± 0.28a-c | 12.94 ± 1.83a-e | 3.27 ± 0.36a-d | 19.58 ± 2.77a-c | 3.56 ± 0.39a |
| JSD3               | 3.05 ± 0.22a-c | 2.54 ± 0.21ab | 9.65 ± 1.05ab | 2.74 ± 0.39a-d | 12.77 ± 3.19a-e | 3.56 ± 0.72a-g | 18.55 ± 4.15a-c | 4.15 ± 0.68a-c |
| JSD5               | 2.96 ± 0.23a | 2.56 ± 0.20ab | 10.13 ± 1.21b-d | 2.97 ± 0.40b-e | 11.81 ± 2.09ab | 3.78 ± 0.76e-h | 17.83 ± 3.16a-c | 4.10 ± 0.64a-c |
| 3MR1               | 3.16 ± 0.29a-d | 2.56 ± 0.21ab | 10.48 ± 1.11b-d | 2.92 ± 0.37b-e | 13.51 ± 1.76e-c | 3.51 ± 0.37a-f | 24.65 ± 5.84d | 3.74 ± 0.39ab |
| JBA6b              | 3.22 ± 0.24b-e | 2.98 ± 0.24de | 9.86 ± 1.11a-e | 3.03 ± 0.39d-f | 14.30 ± 2.26e-f | 3.64 ± 0.54b-g | 20.45 ± 4.33a-d | 4.01 ± 0.64a-c |
| JPD2               | 3.25 ± 0.31c-e | 2.80 ± 0.22c | 10.36 ± 1.35b-d | 2.98 ± 0.36c-e | 13.50 ± 1.96b-e | 3.67 ± 0.43c-h | 19.39 ± 4.75c-e | 3.83 ± 0.63ab |
| KPN4               | 3.27 ± 0.37c-e | 2.98 ± 0.30de | 10.91 ± 1.46cd | 3.20 ± 0.35ef | 14.01 ± 2.51ef | 3.96 ± 0.53f-h | 22.02 ± 4.64edc | 4.17 ± 0.56c-e |
| DRI4a              | 3.61 ± 0.39f | 3.22 ± 0.30f | 9.75 ± 1.38ab | 3.03 ± 0.44d-f | 13.62 ± 2.06e-c | 3.91 ± 0.65f-h | 18.91 ± 3.56c-e | 4.23 ± 0.45bc |
| 3JC6               | 3.56 ± 0.27f | 2.99 ± 0.29e | 10.27 ± 1.05b-d | 2.57 ± 0.27a-f | 11.29 ± 1.18a | 3.13 ± 0.47a-f | 17.89 ± 2.26a-c | 3.58 ± 0.54a |
| JJB22              | 3.10 ± 0.24a-d | 2.47 ± 0.17a | 10.33 ± 1.02b-d | 2.64 ± 0.38ab | 13.84 ± 2.58de | 3.58 ± 0.77a-g | 19.91 ± 4.39a-c | 3.83 ± 0.81ab |
| JS1                | 2.93 ± 0.29a | 2.48 ± 0.23a | 10.39 ± 1.79b-d | 2.97 ± 0.44b-e | 12.79 ± 2.09a-e | 3.69 ± 0.77d-h | 20.92 ± 3.60b-d | 4.15 ± 0.94c-a |
| KSA5               | 3.32 ± 0.29de | 2.63 ± 0.19ab | 10.24 ± 1.32b-d | 2.79 ± 0.32a-d | 12.63 ± 1.76e-f | 3.36 ± 0.44a-c | 19.75 ± 4.11c-e | 3.83 ± 0.48ab |
| JMR2o              | 3.46 ± 0.39ef | 2.82 ± 0.31c-e | 9.78 ± 1.10ab | 3.32 ± 0.55gf | 12.15 ± 1.45a-d | 4.10 ± 0.47h | 16.79 ± 3.22ab | 4.25 ± 0.65bc |
| JMR2c              | 3.42 ± 0.36ef | 2.99 ± 0.25e | 10.34 ± 1.11b-d | 3.20 ± 0.35f-e | 13.80 ± 2.45f-e | 4.11 ± 0.60f-h | 21.75 ± 5.48edc | 4.58 ± 0.53c |
| DBA5               | 3.45 ± 0.47ef | 2.81 ± 0.31c-e | 10.83 ± 1.47c-d | 3.20 ± 0.52f-e | 13.46 ± 2.28e-f | 4.01 ± 0.53gh | 20.61 ± 5.60a-d | 4.26 ± 0.53bc |
| Avg.               | 3.24 ± 0.30a | 2.73 ± 0.24a | 10.14 ± 1.32a | 2.94 ± 0.47a | 12.94 ± 2.30a | 3.61 ± 0.65a | 19.66 ± 4.47ab | 3.96 ± 0.97a |

* Numbers with different letters represent significant difference within isolates of the same species according to Tukey HSD test (p < 0.01).
** Bolded numbers with different letters represent significant difference in micromorphology among species according to Tukey HSD test (p < 0.01).

3.6. Ehrlich Test

Ehrlich reagent reacted with 12 out of 18 *P. expansum* isolates as yellow rings were detected and six isolates formed faint violet rings. All *P. crustosum* isolates formed faint yellow to yellow rings and *P. solitum* isolate also formed a yellow ring. Observed reactions indicated that six *P. expansum* isolates produced cyclopiazonic acid, while the other 12 *P. expansum* isolates, and all *P. crustosum* isolates and *P. solitum* isolate produced other alkaloids.

3.7. *Penicillium Multilocus Phylogeny*

The ITS, *BenA*, *CaM*, and *RPB2* multiple sequence alignments contained 561, 448, 521, and 971 nucleotides, of which 9, 40, 53, and 90, were parsimony informative, respectively. Different nucleotide substitution models were used for individual ML analyses: Tamura’s 3-parameter model (T92) for ITS, Kimura’s 2-parameter model (K2) for *BenA* and *CaM*, and Kimura’s 2-parameter model with a discrete gamma distribution (K2 + G) for *RPB2*. MP analyses of ITS, *BenA*, *CaM*, and *RPB2* resulted each in 10 equally most parsimonious
trees. ML and MP analyses, based on the same single locus, produced trees with identical topologies. Comparing the trees inferred from different loci, the most congruent were those based on BenA and CaM. The combined dataset of the concatenated three single locus alignments (BenA, CaM, and RPB2) contained 1928 characters, of which 176 were parsimony informative, and the combined dataset of the concatenated four single locus alignments contained 2498 characters, of which 169 were parsimony informative. Multilocus phylogenetic trees constructed by ML method, using K2 + G, had the same topology as MP trees, and were consistent with topology of RPB2 tree. MP analysis resulted in 10 equally most parsimonious trees. Selected phylogenetic trees are presented in Figures 6 and 7.

Each phylogenetic analysis conducted in this study clearly separated P. expansum and P. crustosum. No difference within species was observed in phylogenetic analysis based on the ITS region. P. expansum isolates were (sub) divided into two well-supported clades based on BenA and CaM phylogeny, corresponding to two groups separated by macromorphology. RPB2 and multilocus phylogeny confirmed the separation of P. expansum isolates into two groups with the exception of three isolates that were relocated from one group to another (Table 5).

P. crustosum isolates resided in one clade based on ITS, BenA and CaM phylogeny. RPB2 and multilocus phylogeny revealed three groups within P. crustosum clade, all of which received bootstrap support above 60% in ML analyses based on both datasets. P. solitum isolate was grouped with reference isolates of P. solitum into one clade based on each phylogenetic analysis conducted in this study, except the one based on ITS which could not separate P. solitum from some similar Penicillium species. P. solitum clade received the highest bootstrap support (100%) in multilocus phylogeny, followed by RPB2 phylogeny (99%). RPB2 has shown to be the most variable region in all obtained Penicillium spp.

3.8. Virulence Phenotypes in Apple Fruit

Representative P. expansum and P. crustosum isolates caused typical blue mold symptoms in all apple cultivars evaluated. Lesions were soft, watery, light to medium brown, with or without concentric zones, with smooth or irregular margins, and with blue green conidial tufts present mainly around the inoculation cite. In some cases (i.e., P. crustosum isolates JBA8a, MRI4) on apple fruit ‘Granny Smith’ a yellow ring was observed around decayed area. On cross sections of inoculated apple fruit, differences between the decay caused by P. expansum and P. crustosum, were observed. P. crustosum isolates caused slightly darker color of the decayed tissue in all cultivars and sporulated both around the wound and in the apple flesh of ‘Red Delicious’. P. expansum isolates formed lightly coloured decayed areas and scarcely sporulated only around the inoculation cite, not in the apple flesh. In apple fruit inoculated with P. solitum, tissue darkening was observed only around inoculation cite and a yellow ring was present (Figure 8).

Based on lesion size, P. expansum was significantly more virulent than P. crustosum on all three apple cultivars (‘Golden Delicious’, ‘Red Delicious’, and ‘Granny Smith’), 7 dpi ($p = 0.003$, $p < 0.0001$, $p = 0.001$) (Figure 9a) and 9 dpi ($p < 0.0001$, $p = 0.001$, $p = 0.002$) (Figure 9b). Nevertheless, virulence of each species depended on the apple cultivar. P. expansum was the most virulent on ‘Red Delicious’, and produced larger lesions than on ‘Golden Delicious’ ($p < 0.0001$). P. crustosum was equally virulent on ‘Golden Delicious’ and ‘Red Delicious’ (7 dpi, $p = 0.015$). Both species were the least virulent on ‘Granny Smith’. The largest difference in virulence between P. expansum and P. crustosum was observed on ‘Red Delicious’.
Figure 6. Phylogenetic relationships amongst *Penicillium expansum*, *P. crustosum*, and *P. solitum* based on ITS, BenA, CaM, and RPB2. Bootstrap values larger than 60 of the Maximum Likelihood (ML) and Maximum Parsimony (MP) analysis are shown above or below the branches. The tree is rooted with *Penicillium lanosocoeruleum* as an outgroup. Numbers on the branches present bootstrap values obtained for 1000 replicates. Bolded isolates are reference isolates, and bolded isolates with * are ex-type isolates.
Figure 7. Phylogenetic relationships amongst Penicillium expansum, P. crustosum, and P. solitum based on three (BenA, CaM, and RPB2) (a) and four loci (ITS, BenA, CaM, and RPB2) (b). Bootstrap values larger than 60 of the Maximum Likelihood (ML) and Maximum Parsimony (MP) analysis are shown above or below the branches. The tree is rooted with Penicillium lanosocoeruleum as an outgroup. Numbers on the branches present bootstrap values obtained for 1000 replicates. Bolded isolates are reference isolates, and bolded isolates with * are ex-type isolates.

Within species, variability in virulence was observed amongst P. expansum isolates on ‘Red Delicious’ (7 dpi \( p < 0.0001 \)), and amongst P. crustosum isolates on ‘Granny Smith’ (7 dpi, \( p < 0.0001 \)). Cluster analyses based on lesion size, separated three groups within P. crustosum isolates the same way that was determined for colony growth (shown in Figure 5), and it corresponds with three sub clusters obtained via phylogenetic analysis (RPB2 and multilocus). No influence of isolate origin on virulence was observed (statistical data not shown). Two-way ANOVA revealed that both apple cultivar (\( \eta^2 = 0.419 \) for 7 dpi and \( \eta^2 = 0.475 \) for 9 dpi) and species (\( \eta^2 = 0.237 \) for 7 dpi, \( \eta^2 = 0.252 \) for 9 dpi) had a large influence on lesion size. Combined influence of both factors: species and apple cultivar on lesion size was moderate (\( \eta^2 = 0.123 \) for 7 dpi and \( \eta^2 = 0.110 \) for 9 dpi), suggesting that the combination of the most susceptible cultivar and the most virulent pathogen caused rapid disease progressio (‘Red Delicious’ + P. expansum), while disease progressed more slowly in case of less susceptible cultivar and less aggressive pathogen (‘Granny Smith’ + P. crustosum).
Table 5. Placement of *Penicillium expansum* isolates based on different characteristics.

| Macromorphology (Cluster Analysis: Colony Diameter, PDA, MEA, CYA Reverse) | Phylogeny |
| --- | --- |
|  | BenA CaM RPB2 Multilocus |
| I group |  |
| JRad4 * | JRad4 | JRad4 |
| 3JC11 | 3JC11 | 3JC11 |
| 3JC23 | 3JC23 | 3JC23 |
| 3JB13 | 3JB13 | 3JB13 |
| 3SD3 | 3SD3 | 3SD3 |
| 3SD5 | 3SD5 | 3SD5 |
| 3MR1 | 3MR1 | 3MR1 |
| JBA8b | JBA8b | JBA8b |
| JPN2 | JPN2 | JPN2 |
| KPN4 | KPN4 | KPN4 |
| DR14a | DR14a | DR14a |
| JBA8b | JBA8b | JBA8b |
| JPN2 | JPN2 | JPN2 |
| KPN4 | KPN4 | KPN4 |
| DR14a | DR14a | DR14a |
| JBA8b | JBA8b | JBA8b |
| JPN2 | JPN2 | JPN2 |
| KPN4 | KPN4 | KPN4 |
| DR14a | DR14a | DR14a |
| JBA8b | JBA8b | JBA8b |
| JPN2 | JPN2 | JPN2 |
| KPN4 | KPN4 | KPN4 |
| DR14a | DR14a | DR14a |

II group

| 3JB22 | 3JB22 |
| 3S1 | 3S1 |
| KSA5 | KSA5 |
| 3JC6 | 3JC6 |
| JMR2o | JMR2o |
| JMR2z | JMR2z |
| DBA5 | DBA5 |

* Bolded isolates show stable placement in groups based on all characters.

3.9. Principle Component Analysis

PCA analysis separated all 26 tested isolates based on morphological data (micro, macro and growth rate) in three groups (Figure 10a). The total variability of 58.31% was explained by first and second component. Principal component 1 (PC1), accounts for 37.93% of the variance, was mostly positively associated with conidia and metulae width and negatively associated with colony diameter on PDA, CYA and YES. It clearly separates faster growing isolates (left) from those with smaller colonies and with larger conidia or conidiophore parts (right). PC2, which accounts for 20.38% of the variance, is strongly positively associated with colony diameter on MEA and PDA, and metulae width, while is negatively associated with coloration on CYA reverse (red and pink coloration has smaller value than yellow or cream coloration). It separates faster growing isolates firstly on MEA, then on PDA with wider metulae (up) from isolates that have cream or yellow coloration on CYA reverse (down). It also shows the similarity of the second *P. expansum* group to *P. crustosum*. Separation of *P. expansum* isolates into two groups based on morphology was in accordance with phylogenetic separation based on single BenA and CaM loci (Table 5).

PCA analysis grouped 14 representative isolates based on macro- and micromorphology and virulence on ‘Red Delicious’ and ‘Golden Delicious’ at 7 and 9 dpi. The total variability of 61.72% was explained by first and third component. PC1 accounts for 45.83% of the observed variance, and was mostly associated with colony diameter on all tested media, and lesion size on ‘Red Delicious’ at 7 dpi and 9 dpi. It separates more virulent isolates with larger colony growth, from less virulent isolates, which formed smaller colonies. Thus, the majority of *P. expansum* isolates were distinct from *P. crustosum* isolates, and were grouped on the right side of the graph, except from two isolates DBA5 and JMR2z. PC3, which accounts for 15.89% of the variance, is strongly and positively associated with metulae and rami width, and separates isolates with wider metulae and rami (up) from isolates that formed narrower metulae and rami (down) (Figure 10b).
Penicillium expansum and P. crustosum mean lesion diameter (mm ± SE) on inoculated apple fruit ‘Golden Delicious’, ‘Red Delicious’ and ‘Granny Smith’ at 7 dpi (a) and 9 dpi (b). Numbers with different letters represent significant difference in lesion diameter amongst species according to Tukey HSD test ($p < 0.01$).

Figure 8. Blue mold decay caused by *Penicillium expansum* (A), *P. crustosum* (B), and *P. solitum* (C) on different apple fruit cvs. 9 dpi at 24 ± 2 °C and control (D).

Figure 9. *Penicillium expansum* and *P. crustosum* mean lesion diameter (mm ± SE) on inoculated apple fruit ‘Golden Delicious’, ‘Red Delicious’ and ‘Granny Smith’ at 7 dpi (a) and 9 dpi (b). Numbers with different letters represent significant difference in lesion diameter amongst species according to Tukey HSD test ($p < 0.01$).
Figure 10. Projection of Penicillium expansum and P. crustosum isolates based on: (a) first and second principal component, according to principal component analysis (PCA) of isolate characteristics (micro, macromorphology, and colony growth). *P. expansum* isolate groups are purple, while *P. crustosum* isolate group is green (DRI3 is *P. solitum* isolate); (b) first and third principal component, according to principal component analysis (PCA) of isolate characteristics (micro, macromorphology, colony growth, and virulence). *P. expansum* isolate group is turquoise, while *P. crustosum* isolate group is green.

### 4. Discussion

Data regarding the incidence of blue mold decay, diversity of pome fruit hosts, variation in fungal virulence, and distribution of *Penicillium* spp. causing postharvest rot resulted in confirmatory and novel findings. *Penicillium expansum* and *P. crustosum* caused blue mold on stored apple and pears which is congruent with previous studies [20–24,55],
while blue mold on quince caused by *P. expansum*, *P. crustosum* and *P. solitum* is a novel find in Serbia. Additionally, *P. crustosum* has not been identified on medlar nor quince, indicating two new hosts for this blue mold causing species. Occurrence of *P. solitum* on quince has not been previously reported in the literature, and multiple gaps regarding various pome fruit hosts and causative *Penicillium* spp. were unknown. Hence, novel discoveries resulted from the comprehensive survey, virulence assessment and accompanying morpho-genetic characterization of *Penicillium* spp.

Incidence regarding the most common causal agent of blue mold on pomaceous fruit in Serbia showed that *P. expansum* was most prevalent (91.64%), followed by *P. crustosum* (7.29%) which were detected on all pome fruit examined, with *P. solitum* having the lowest occurrence. A survey of apples and pears with blue mold symptoms from Oregon and Washington State showed that several *Penicillium* spp. (*P. auarantiogriseum*, *P. commune*, *P. solitum*, *P. verrucosum*, and *P. expansum*) caused blue mold [14]. Another study conducted in British Columbia showed that *P. brevicompactum*, *P. crustosum*, and *P. expansum* were isolated from apple fruit with blue mold symptoms [15]. In Uruguay, it was found that *P. expansum* and *P. solitum* were the main causal agents of apple and pear decay in storage [13]. Our findings mirror what has been shown over different parts of the world regarding *Penicillium* spp. diversity, and it appears that regardless of geographical location, *P. expansum* is most prevalent and aggressive of all blue mold fungi obtained from stored pomes. Many factors may underpin *P. expansum*’s cosmopolitan status including but not limited to: e.g., the pathogens genomic plasticity, its ability to suppress, inactivate and overcome preformed and induced host plant defences, deployment of an arsenal of virulence factors, and the biosynthesis of small molecules that aid in decay [56]. Future comparative multomics investigations with the isolates from this study will be used to ascertain the underlying mechanism(s) that facilitate *P. expansum*’s broad ranging success as a necrotrophic fungal pathogen.

*Penicillium expansum* is the most virulent *Penicillium* species and is well adapted to infect, colonize and decay apple fruit [9]. Our findings agree with Morales et al. [57] that there is low variability in *P. expansum*’s ability to colonize apple flesh, however variation in some features (e.g., macromorphology and phylogeny) was observed. Also, we support the suggestion of Morales et al. [57] that factors involved in fruit colonization seem to be constant in each *P. expansum* population. In this study *P. expansum* was found to be more virulent than *P. crustosum* on all three apple fruit cultivars, which was also observed in other studies on stored apple and pear fruits [6,14,58]. Differences in *P. expansum* and *P. crustosum* virulence in apple could be explained via metabolic flux in the host during the infection process. *P. expansum* causes more intense and dynamic metabolic changes which helps the pathogen overcome host defenses and results in decreased pools of phenolics and glutathione compared to *P. crustosum*-mediated decay [59,60]. It is well known that high phenolic content is important for apple fruit defense against blue mold decay [61–63]. This difference is also reflected in the array of observed virulence phenotypes in apple fruit conducted during this study. Even though *P. crustosum* is a less well adapted pathogen compared to *P. expansum*, it caused decay in apple fruit, and is supported by its ability to abundantly sporulate in the wound. Together, these factors allow *P. crustosum* to be classified as the second most important *Penicillium* species found to cause apple fruit decay. However, both *P. expansum* and *P. crustosum* were least virulent on ‘Granny Smith’ and it’s hypothesized that this could be due to differences in flavanol and glutathione content, which is higher in this cultivar than others [64,65]. Additionally, a yellow ring surrounding the infected area was primarily observed in ‘Granny Smith’ infected with *P. crustosum*. Interestingly, a similar reaction was observed on immature and commercially mature ‘Golden Delicious’ fruit infected with an incompatible apple fruit pathogen *P. digitatum* [56]. Both infection and wounding activate H$_2$O$_2$ accumulation to generate high ROS levels which can damage chloroplasts, resulting in the yellow-colored ring, reminiscent of the same phenomenon we observed during our study [67,68].
Since *P. expansum* and *P. crustosum* were found to be the most dominant causal agents of blue mold in Serbia, the isolates from different origins were subjected to detailed morphological characterization. We observed that YES was the most favourable media for the growth of both species, which agreed with descriptions for *P. expansum* and *P. crustosum* by Pitt and Hocking [69] and Frisvad and Samson [18]. Interestingly, two distinct groups of *P. expansum* were observed based on colony growth, presence/absence of yellow colony reverse on PDA and MEA, and CYA reverse. The first group had: faster growing colonies, cream reverse on PDA and MEA, and salmon pink to red CYA reverse while the second group had: slower colony growth, yellow reverse on PDA and MEA, and variable colour of CYA colony reverse (yellow, cream or red). Variability and correlation of morphological and genetic characteristics was observed for some other *Penicillium* species such as *P. glabrum*. Barreto et al. [70] noted high intraspecific variation of *P. glabrum* from cork in micro-and macromorphology and extrolite profiles, which was supported by partial β-tubulin and calmodulin sequence analyses. Similarly, variability of *P. glabrum* isolates from onion bulbs was observed via molecular (BenA), macro- and micro-morphological and pathogenic characters and these features were correlated [49].

Examination of the *Penicillium* spp. isolates using multiple loci (ITS, BenA, CaM, RPB2) largely agreed with the morphological findings to reveal three main species of blue mold fungi from four different pome fruit hosts. While several distinct *Penicillium* spp. were isolated and characterized, we observed the presence of multiple, well supported subgroups for *P. expansum* based on individual and concatenated loci. Our data show that two well supported subclades, congruent with morphological data, were present for *P. expansum* via BenA, CaM, and concatenated loci. While the consequence for this observation is unclear, it is possible that *P. expansum* is undergoing genetic change(s) that accompany the observed morphological differences that function in ecological, biological and or niche specialization. Despite the biological consequence, our findings are congruent with the literature that the BenA is amongst the most stable and of utmost phylogenetic value for *Penicillium* speciation [50]. Houbraken et al. [42] indicate that subspecific levels such as subspecies, varieties, forma specialis etc. should not be used in a formal taxonomic manner. While their taxonomic value remains debatable, a deeper look into the various subspecies via comparative approaches (e.g., metabolite profiles, genomic organization, and secondary metabolic gene cluster arrangement) will likely yield an abundance of fundamental information to ascertain the biological basis for these observations.

While *P. expansum* and *P. crustosum* were the most prevalent blue mold fungi obtained from pome fruits, isolate origin did not impact the observed morphological, genetic and pathogenic features evaluated in the current study. This is in contrast with what Sanzani et al. [71] who showed genetic and morphological variation amongst *P. expansum* and *P. crustosum* isolates, based on a single locus BenA, which grouped the isolates based on the origin of the host (confirmed by the High-Resolution Melting). Thus, they proposed that host specialization had occurred amongst the isolates of the same species. However, we didn’t observe this pattern. It is possible that our isolates are not as evolved and or that the isolates from Italian collection possess more genetic variation at loci that mediate outcrossing and or recombination. A detailed genome-wide study conducted by Julca et al. [72] supports our finding in that they showed a relatively high sequence divergence of *P. expansum*, which did not correlate with the geographical distance between isolates. This indicates that isolates may have diverged a long time ago but that the geographical structure of the populations may have been influenced by migration. *P. expansum* has the capacity to undergo both meiotic and mitotic recombination and exhibits large genetic diversity, despite its primary asexual mode of reproduction. This is further supported by genome mining studies that show *P. expansum* is heterothallic. Hence, there is potential that cryptic mating amongst divergent strains may have contributed to the observed patterns of genomic variation [72].

Our two-year survey highlights new morphological, and phylogenetic aspects of *Penicillium* spp. from stored pome fruits in Serbia, has filled several knowledge gaps,
brought forth new findings concerning fungal virulence, and uncovered interesting observations to further explore. Our study showed for the first time that *P. crustosum* and *P. solitum* infect quince, and that medlar is a new host of *P. crustosum*. Additionally, *P. expansum* is a new pathogen of quince in Serbia. Rigorous phylogenetic and morphological investigations show that there are three species that predominate pome fruit storage and that *P. expansum* has two distinct phylogenetic subgroups and *P. crustosum* has three according to various loci. Consistent with previous findings we have shown that in general, *P. expansum* is the most commonly isolated and aggressive blue mold species followed by *P. crustosum* and the least prevalent and weakest is *P. solitum*. Our morphological and phylogenetic findings suggest that *P. expansum* is changing at multiple levels and we propose future omics-based and functional genetic studies to understand the basis for the observed differences within *P. expansum* sub-clades.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7121019/s1, Table S1: List of *Penicillium* spp. isolates obtained in this study.

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