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Phytophthora Species from Xinjiang Wild Apple Forests in China

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Received: 28 August 2019; Accepted: 17 October 2019; Published: 21 October 2019

Abstract: *Phytophthora* species are well-known destructive forest pathogens, especially in natural ecosystems. The wild apple (*Malus sieversii* (Ledeb.) Roem.) is the primary ancestor of *M. domestica* (Borkh.) and important germplasm resource for apple breeding and improvement. During the period from 2016 to 2018, a survey of *Phytophthora* diversity was performed at four wild apple forest plots (Xin Yuan (XY), Ba Lian (BL), Ku Erdening (KE), and Jin Qikesai (JQ)) on the northern slopes of Tianshan Mountain in Xinjiang, China. *Phytophthora* species were isolated from baiting leaves from stream, canopy drip, and soil samples and were identified based on morphological observations and the rDNA internal transcribed spacer (ITS) sequence analysis. This is the first comprehensive study from Xinjiang to examine the *Phytophthora* communities in wild apple forests The 621 resulting *Phytophthora* isolates were found to reside in 10 different *Phytophthora* species: eight known species (*P. lacustris* being the most frequent, followed by *P. gonapodyides*, *P. plurivora*, *P. gregata*, *P. chlamydospora*, *P. inundata*, *P. virginiana*, and *P. cactorum*) and two previously unrecognized species (*P.* sp. CYP74 and *P.* sp. forestsoil-like). The highest species richness of *Phytophthora* occurred at BL, followed by XY. *P. lacustris* was the dominant species at BL, XY, and JQ, while *P. gonapodyides* was the most common at KE. In the present paper, the possible reasons for their distribution, associated implications, and associated diseases are discussed.

Keywords: *Phytophthora*; diversity; wild apple forest; decline

1. Introduction

Xinjiang wild apple (*Malus sieversii* (Ledeb.) Roem.), the wild ancestor of the domesticated apple, is mainly distributed in the Tian Shan mountains in Central Asia, including the Ili River Valley in northwest China's Xinjiang Uygur Autonomous Region and southeast and east Kyrgyzstan [1–3]. It is the dominant species in the relict wild fruit wood forests of inner Eurasia and is protected as a vulnerable species among the endangered rare germplasm resources of China [4–6]. However, the wild populations of *M. sieversii* have experienced a dramatic decrease in recent years, and symptoms of the decline can be observed in many wild apple forests. Affected trees show higher canopy loss, branch dieback, bark and cambium necrosis, and growth reduction. Similar to other countries in Central Asia, several abiotic and biotic factors negatively affect the health status of wild apple forests in China [7], including environmental and climate impacts, insect pests, cambium feeders such as *Agrilus mali* Matsumura, and infection by pathogenic fungi [8–13] (Figure 1).

Figure 1. Declining symptoms of wild apple forests in Xinjiang.

The presence of *Phytophthora* species in forests and natural ecosystems is considered to be an important biotic factor responsible for the decline, dieback, and mortality of trees [14]. Belonging to the class Oomycetes, or "water molds", in the kingdom Chromista, these fungus-like organisms can cause root rot, bark cankers, and diseases leading to the decline and dieback of a wide range of plant species worldwide [15]. Over the past two decades, numerous surveys have shown that many known and previously unknown *Phytophthora* species are present in a variety of natural and semi-natural forests and river systems in Europe, America, Australia, South Africa, and more recently, Asia [16]. Some of these *Phytophthora* species, including *P. cactorum*, *P. cinnamomi*, and *P. plurivora*, have shown strong involvement in the decline and dieback of forests, while the exact role in forest ecosystems of many other species, such as *P. cryptogea*, *P. chlamydospora*, and *P. gonapodyides*, is unclear [16]. In a recent study, *P. plurivora* was found to cause damage to the fine roots and stems of *M. sieversii* in the declining wild fruit forests of Xinjiang Province, China [17]. However, the findings described in that report were based on a limited number of samples. Furthermore, the distribution and ecological roles of *P. plurivora* and other *Phytophthora* species in wild apple forests are still unknown.

In June to October 2016–2018, a survey of *Phytophthora* diversity was performed at four plots in wild apple forests on the northern slope of Tianshan Mountain in Xinjiang using baiting assays from rhizosphere soil, streams, and canopy drip. This study presents the results of this survey related to the *Phytophthora* species associated with the decline or dieback of *Malus sieversii*.

2. Material and Methods

2.1. Study Area

The study was carried out in wild apple forests on the northern slopes of Tianshan Mountain in Xinjiang, China. We chose Xin Yuan (XY) (83°33′ E, 43.25′ N), Ba Lian (BL) (82°50′ E, 43°15′ N), Ku Erdening (KE) (82°51′ E, 43°13′ N), and Jin Qikesai (JQ) (83°25′ E, 43°18′ N) as the studied trap plots, as these locations are where the wild apple trees mostly live [18] (Figure 2). In these four plots, the decline of wild apple trees in XY is the most serious. A total of 10 stream sites that flow though the declining wild apple trees were set at each of the 4 areas; 10 canopy drip sites under the declining wild apple trees were set at each of the 4 areas. Fifteen soil sites under the declining trees were set at XY and at BL. In total, 40 stream sites, 40 forest sites, and 30 soil sampling sites were set in these 4 plots to investigate the presence of *Phytophthora* in the wild apple forests.

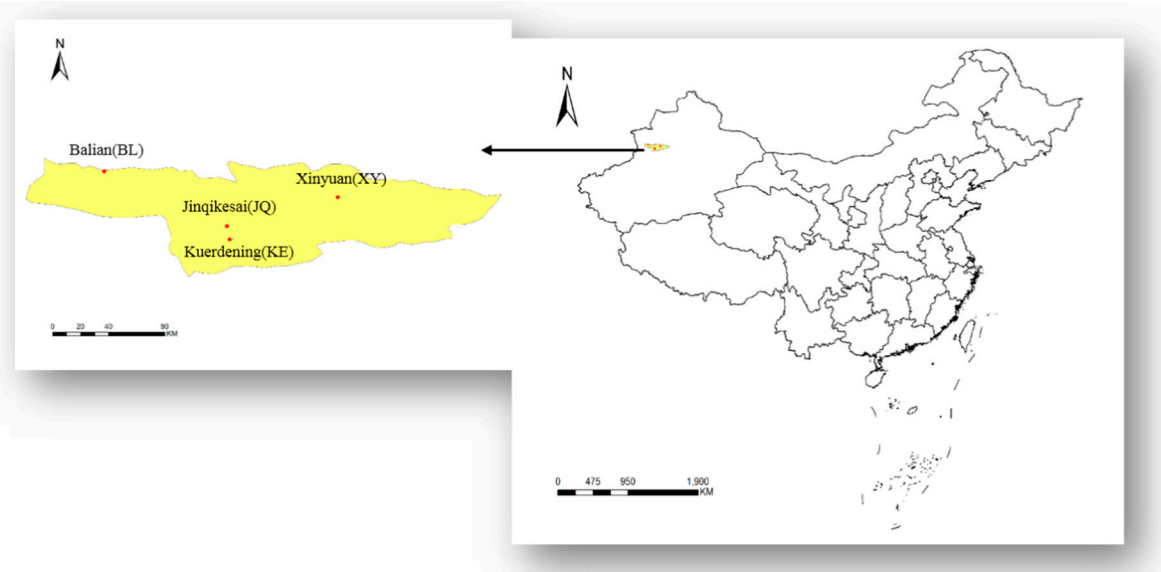

Figure 2. The four plots located in Xinyuang, Gongliu county, China.

2.2. Sampling and Phytophthora Isolation

This research used stream, canopy drip, and soil sampling baiting [19–22] at the 4 plots from 2016 to 2018. All isolates of *Phytophthora* spp. were recovered from sites by baiting with leaves of wax (*Fraxinus chinensis* Roxb). The baiting leaves were placed in the surveyed sites for 1 week, retrieved, and brought to the laboratory from June to October each year. Pieces of approximately 2 mm^2 were cut from the margins of the brown spots and plated in CARP+. Colonies of suspected *Phytophthora* species growing from plated baits were transferred to CARP (CARP+ without Benlate and hymexazol) to confirm purity and then to CMA for characterization and storage [23].

2.3. Classical Identification of Isolates

Morphospecies were first identified by colony patterns in V8 agar (V8A). Colony growth patterns of 7 day old cultures grown at 20 °C in the dark in V8A and morphological features of sporangia, oogonia, antheridia, chlamydospores, hyphal swellings, and aggregations were studied, described, and photographed. The formation of sporangia was stimulated by flooding small V8A disks (0.5 mm diameter) with sterile water at room temperature, and this was observed by microscope after 12, 24, 48, and 72 hours. The results were compared with those of known isolates and species descriptions present in the literature [24].

2.4. Molecular Identification of Isolates

DNA isolation and amplification was carried out. For all *Phytophthora* isolates obtained in this study, mycelial DNA was extracted from pure cultures grown in corn meal agar (CMA). DNA extraction and amplification were performed in accordance with Huai et al. [25], using the primer pairs ITS6 (5′-GAA GGT GAA GTC GTA ACA AGG-3′) [26] and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) [27] to amplify both ITS1 and ITS2 regions, including the 5.8S rDNA. The PCR reaction was conducted in a 25 µL reaction mixture consisting of 12.5 µL of 2× Taq Master Mix, 9.5 µL of double-distilled H_2O (both supplied by TIANGEN Biotech, Beijing, China), 0.75 µL of rDNA internal transcribed spacer (ITS) primers or 0.25 µL of Btub or EF1a primer (all primers were sourced from Sangon Biotech, Shanghai, China), and 1.5 µL of DNA template. The PCR thermal cycling program was as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles consisting of denaturation at 95 °C for 1 min, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min.

Sequence data were initially assembled using STADEN Package 2.0.0. and compared with other closely related species, including some undescribed taxa obtained from GenBank [28].

2.5. Phylogenetic Analysis

Sequences were aligned using MEGA version 6.0. No further editing was done on the alignment to ensure reproducibility and to prevent the introduction of bias. Sequences were concatenated for phylogenetic analyses. Isolates represented each of the known species; the undescribed taxa obtained in this study were compiled into a single data set, and Maximum Likelihood (ML) analysis was performed using MEGA version 6.0. The bootstrap support values were obtained with 1000 bootstrap replicates for each. To ensure general reproducibility, the analysis was repeated using MrBayes to build a Bayesian tree. DANMAN 8 was also used to align multiple sequences to compare the sequences of the same species.

3. Results

3.1. Phytophthora Species Identification

In total, 621 *Phytophthora* isolates were recovered from this study: 261 from BL, 199 from XY, 121 from KE, and 40 from JQ. Most isolates were recovered from streams and rivers. A total of 597 isolates were from stream baiting, 15 were from soil sampling, and nine were from canopy drip.

Ten morphospecies were identified on the basis of colony patterns and micromorphological features. The features of the sporangium were recorded and photographed. There were two unknown *Phythphthora* taxa. One was *P.* sp. CYP74, which is heterothallic, and no oogonia were observed. Another one was *P.* sp. forestsoil-like which is self-sterile. Species identification was confirmed by comparing ITS rDNA sequences. ITS sequence data were obtained for all isolates, and their identities were confirmed by conducting a BLAST search in GenBank. The results of morphological observations were consistent with ITS analysis; 578 isolates were of eight known species, which, in order of frequency, were *P. lacustris*, *P. gonapodyides*, *P. plurivora*, *P. gregata*, *P. chlamydospora*, *P. inundata*, *P. virginiana*, and *P. cactorum*. In addition, 43 isolates of two unknown species were identified; in order of frequency, these were *P.* sp. CYP74 and *P.* sp. forestsoil-like (Table 1).

Table 1. The information of 621 *Phytophthora* strains relating to clades, species, methods, numbers, and plots including Xin Yuan (XY), Ba Lian (BL), Ku Erdening (KE), and Jin Qikesai (JQ).

Clade	Species	Plot	Method	Number
clade1a	*P. cactorum*	BL	canopy drip	1
clade2c	*P. plurivora*	BL	soil	1
		XY	soil	2
		XY	stream	3
		KE	stream	5
clade6b	*P. lacustris*	BL	stream	175
		BL	canopy drip	2
		BL	soil	4
		XY	stream	84
		XY	canopy drip	1
		XY	soil	1
		KE	stream	48
		JQ	stream	28
	P. gonapodyides	BL	stream	56
		XY	stream	74
		XY	canopy drip	5
		XY	soil	3
		KE	stream	63
		JQ	stream	7
	P. chlamydospora	BL	stream	3
		XY	stream	3

Table 1. *Cont.*

Clade	Species	Plot	Method	Number
	P. gregata	BL	stream	2
		XY	stream	4
		XY	soil	1
	P. inundata	XY	stream	1
	P. sp. CYP74	BL	stream	13
		XY	stream	15
		XY	canopy drip	2
		KE	stream	3
		JQ	stream	2
	P. sp. forestsoil-like	BL	stream	3
		KE	stream	2
		JQ	stream	3
clade9a	*P. virginiana*	BL	stream	1

3.2. Phylogenetic Analysis

The aligned ITS dataset consisted of approximately 913 characters. Phylogenetic analysis of Bayes and ML trees revealed sequences that were grouped within four of the 10 main clades of the genus *Phytophthora* [29,30] ascribed to 10 different species (Table 2). Several isolates corresponded to known species as follows: *P. cactorum*—clade 1; *P. plurivora*—clade 2; *P. lacustris*, *P. gonapodyides*, *P. chlamydospora*, *P. gregata* and *P. inundata*—clade 6; *P. virginiana*—clade 9.

Table 2. GenBank number and rDNA internal transcribed spacer (ITS) clade of 10 *Phytophthora* species in Xinjiang wild apple forests.

Species	Isolate No.	ITS Clade	Genbank Number
Phytophthora cactorum	8BLL3	1	MN175469
Phytophthora plurivora	1KEX3(6)	2	MN175458
Phytophthora lacustris	4GLX9(3)	6	MN175455
	8KEX3(2)	6	MN175463
Phytophthora gregata	9XYX6(5)	6	MN175456
	7XYT6(2)	6	MN175459
Phytophthora gonapodyides	9XYX7(2)	6	MN175457
	2KEX5(5)	6	MN175462
Phytophthora sp. CYP74	1KEX9(1)	6	MN175460
	15XYX2(1)	6	MN175465
Phytophthora chlamydospora	9XYX5(4)	6	MN175461
	5BLX9(2)	6	MN175466
Phytophthora sp. forestsoil-like	1BLX1(7)	6	MN175464
	8JQX4(1)	6	MN175468
Phytophthora inundata	15XYX3(1)	6	MN209784
Phytophthora virginiana	1BLX1(3)	9	MN175467

The phylogenetic analysis revealed two undescribed *Phytophthora* taxa, *P.* sp. CYP74 and *P.* sp. forestsoil-like, both from clade 6 (Figure 3). *P.* sp. CYP74 was found to be similar to *P. mississippiae* and *P. ornamentata*. Compared with the isolate *P.* sp. CYP74, the ITS sequence identity of *P. mississippiae* and *P. omamentata* was shown to be 99.66%. *P.* sp. forestsoil-like was found to be similar to *P.* sp. forestsoil-like (TW55), which was reported in Taiwan [24], with an ITS sequence identity of 96.38%.

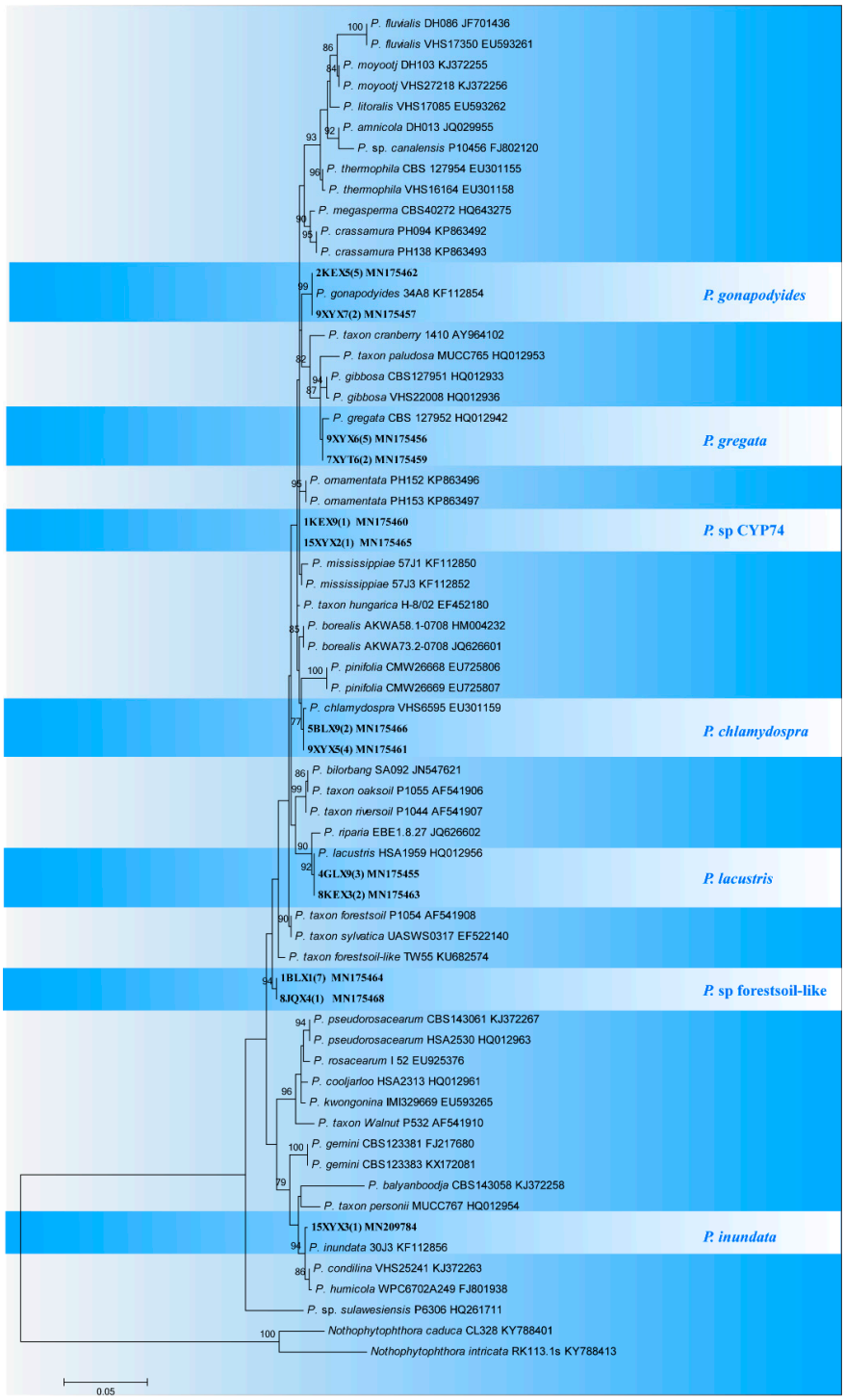

Figure 3. A phylogram based on ITS sequence data indicating the placement of clade 6 and the undescribed *Phytophthora* taxa recovered in this study. The topological structures of Bayes and Maximum likelihood (ML) trees are the same. Bootstrap support is given above the line. Numbers above the branches represent the bootstrap support based on the maximum likelihood analysis.

3.3. The Distribution of Phytophthora Species

In this research, four plots with varying *Phytophthora* diversity were investigated (Figure 4). Ba Lian (BL), with nine species, had the greatest diversity, and Jin Qikesai (JQ) had the least diversity with five species. *P. lacustris* was the most widespread species and the main species at JQ and BL (70% and

69%). It also accounted for the largest proportion at XY, followed by *P. gonapodyides*. Overall, these two species were the most common in the wild apple forests. The third most common species, with 35 strains, was *P*. sp. CYP74; this species was baited at all plots and was predominantly found at XY and BL. *P. cactorum* and *P. virginiana* were only found at BL, and *P. inundata* was only found at XY.

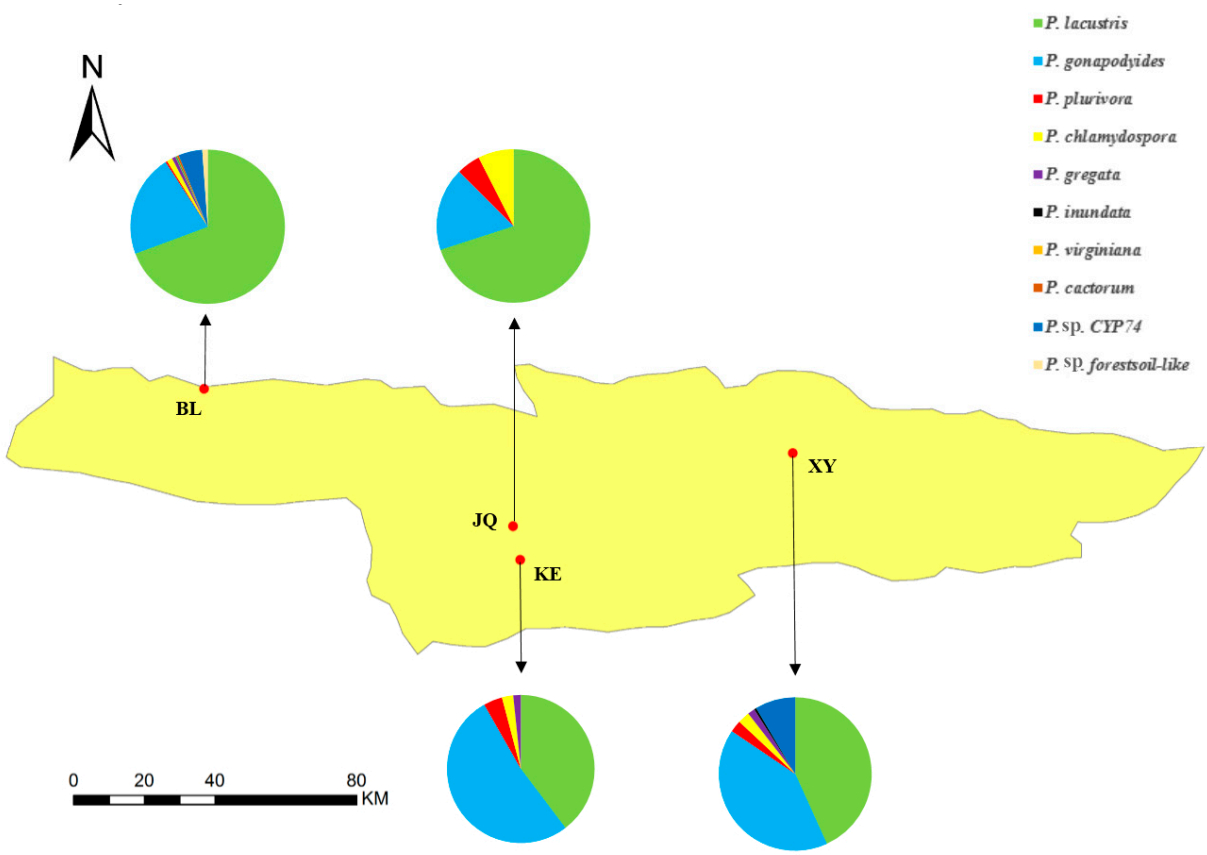

Figure 4. The *Phytophthora* species distribution at four plots.

4. Discussion

The results of this study provide the first record of the broad range of *Phytophthora* spp. associated with the wild apple forest ecosystem in Northwest China. Ten *Phytophthora* species belonging to clades 1, 2, 6, and 9 were isolated in the wild apple forests, including eight species reported for the first time in Xinjiang and two previously unrecognized species. *P. cactorum*, *P. plurivora*, *P. lacustris*, *P. gonapodyides*, *P. gregata*, and *P.* sp. CYP74 were caught in the forest stands. *P. plurivora*, *P. lacustris*, *P. gonapodyides*, *P. chlamydospora*, *P. gregata*, *P. inundata*, *P.* sp. CYP74, *P.* sp. forestsoil-like, and *P. virginiana* were caught in the natural rivers. The present work indicates the diversity and distribution of *Phytophthora* in Xinjiang wild apple forests.

From clade 6, *P. lacustris* and *P. gonapodyides* were caught in 4 plots from June to October. The number of these two species took up 88.6% of all *Phytophthora* species in this survey. In particular, *P. lacustris* represented more than half of the total number of strains. *P. lacustris* and *P. gonapodyides* were obtained from canopy drip samples, soil samples, and mostly stream samples. These species often co-exist in river systems in the temperate regions of North America, Europe, and Asia [20,31,32]. *P. lacustris*, which like *P. gonapodyides* belongs to clade 6, is widely distributed globally. Initially identified as a saprotroph that infects plant detritus, it has now been shown to cause significant damage to fine roots and weak-to-moderate bark lesions in *Alnus glutinosa* and *Prunus persica* in Portugal, Italy, and Turkey, among other places [33–37]. Samples in previous research on this species were from soil, trees, and roots, while in the present study, they were mostly taken from streams, with a few collected by

canopy drip and from the soil by baiting. In the present study, *P. lacustris* was acquired from all four site plots, demonstrating that a large number of *P. lacustris* live in the wild apple forests of Xinjiang, especially in the riparian habitats of streams. *P. gonapodyides* was described in 1927 as a global species, appearing in almost every *Phytophthora* survey and demonstrating weak pathogenicity [32,38–43].

P. gregata was obtained from stream samples at BL in July and stream, soil samples at XY in September. It was reported in China in 2013 by stream baiting [20] and was shown to cause significant reduction of shoot and root growth but was not found to kill plants [44].

The present survey is the first report of *P. inundata* in China but was previously reported as a pathogen of shrubs and trees in Europe and South America [45] and the cause of Viburnum latent infection in Australia and Virginia [46]. All *Phytophthora* species have the potential to disturb natural ecosystems, particularly those of exotic origin, provided that environmental conditions are conducive to disease development [14,32,47].

P. chlamydospora was caught at BL in August by stream baiting and stream samples at XY in September. It has been recovered in Europe, North America, Argentina, and Taiwan from cankers on trees, roots, and foliage of horticultural nursery stock [16,24,48,49].

In this research, we baited two undescribed species: *P.* sp. CYP74, which is heterothallic like *P. mississippiae*, and *P.* sp. forestsoil-like, which is self-sterile like *P.* sp. forestsoil-like (TW55), reported in Taiwan in 2017 [24]. Detailed information for these two species will be shown in future studies.

P. plurivora from clade 2 is known to be a serious pathogen of many forest trees, including oak, beech, and *Alnus glutinosa* seedlings. This species can cause dieback and root loss and is most frequently associated with cankers in Europe, North America, and Asia [31–33,50–55]. Via the examination of plant tissues and soil samples, it has been reported to cause cankers in wild apple forests in Xinjiang [17], corroborating its discovery in stream water and soil samples in the present study.

From clade 1, we collected *P. cactorum* at BL by canopy drip in September. First described in 1886, this clade1a species is similar to the notorious pathogenic species *P. infestans*, which can cause damping-off of seedlings, fruits, leaf stems, and roots, as well as collar and crown rot and stem canker on an extremely wide host range of more than 200 species from 160 generas of plants, including many fruits, ornamental plants, and forest trees. It has been reported to be the cause of aerial cankers on European beech trees and is a major problem in apple orchards, causing the death of apple trees [56]. A previous study of staple crops in Xinjiang showed that *P. cactorum* was isolated from the fruits, root crowns, and diseased soils of strawberry, safflower, apple, and pear plants [47]. The present study represents the first time that *P. cactorum* has been found in the forest system in Xinjiang. It may have spread to the forests from nearby farms and is a probable reason for the decline in apple trees [15,57–59].

In clade 9, *P. virginiana* was obtained at BL by stream baiting in July. It was first isolated from irrigation water at several ornamental nurseries in 2013 in Virginia [60]. No pathogenicity of this species has yet been detected.

In this study, all plots were situated in areas of declining wild apple trees. Although the diversity of *Phytophthora* was different at different plots, it may be related to the population density of wild apple forests and human activities. We will set some survey plots in healthy stands and investigate the pathogenicity of these *Phytophthora* species in future studies to confirm their effects on this wild apple forest ecological system and better understand the reasons for its decline.

5. Conclusions

1) This first extensive survey demonstrated 10 *Phytophthora* species in a Xinjing wild apple forest ecosystem. Discussing the potential pathogenicity of these *Phytophthora*, this is a basic study to find out the reasons why the wild apple trees have declined.

2) *P. lacustris* and *P. gonapodyides* are the most widespread species, and BL has the highest number of *Phytophthora* species. Two undescribed species were also detected in this research. These results form a foundation for the study of the genetic diversity of *Phytophthora*.

Author Contributions: Conceptualization, W.Z. and W.H.; methodology, X.X.; formal analysis, X.X.; investigation, X.Z.; resources, H.; writing—original draft preparation, X.X.; writing—review and editing, W.H.; supervision, W.Z.; project administration, W.Z.; funding acquisition, W.Z. and W.H.

Funding: This research was funded by National Key R&D Program of China, grant number 2016YFC0501503 and National Natural Science Foundation of China, No. 31470649. The APC was funded by National Key R&D Program of China, 2016YFC0501503.

Acknowledgments: The authors are grateful to the Zhongsheng Li and Jinlong Zhang for material support, Jiaqing Jiang for strains saved during this work. In particular, thank Krishan Andradige for his kind encouragements and supports.

Conflicts of Interest: The authors declare no conflict of interest.

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