Journal of General Plant Pathology
https://doi.org/10.1007/s10327-018-00836-4

FUNGAL DISEASES

Genetic diversity of *Phytophthora nicotianae* reveals pathogen transmission mode in Japan

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Received: 30 May 2018 / Accepted: 10 September 2018

Abstract

Phytophthora nicotianae is an important soil-borne pathogen in tropical, subtropical and temperate regions. To clarify the genetic diversity of *P. nicotianae* and to understand its mode of transmission in Japan, we developed six new microsatellites markers, consisting of six loci and 39 alleles. In a phylogenetic analysis, 138 isolates, including 125 from Japan and 13 from overseas, were shown to differ, even though some were collected from the same host and location, suggesting that there is no geographic or host plant clustering. Population structure analysis also revealed a highly admixed population of *P. nicotianae* in Japan. Molecular analysis suggested high variance between individuals but no significant differences between populations. Both A1 and A2 mating types were present in the same population, which could be due to high levels of variance between individuals in the population. The absence of geographical structure between populations also suggests that the pathogen is able to migrate from one population to another. We propose that this phenomenon could result from human activities related to the transport of plant and associated agricultural materials.

Keywords Diversity · Microsatellite · *Phytophthora nicotianae* · Population genetics · Population structure

Introduction

As advancements in transportation technology have made global trading easier, the resultant global redistribution of species by human activities has included not only the introduction of beneficial species to new environments, but also the introduction of their associated pathogens. Most crops are in the process of rapid biotic homogenization, which can potentially lead to significant reductions in the genetic variability of the principal crops of many important agricultural nations within the next few decades (Bebber et al. 2014). Such losses pose a threat to crop species because the evolution of a new virulent variant of a pathogen could result in high losses and poor yields if the pathogen spreads rapidly. The near extinction of the Gros Michel banana in the 1950s is a good example: the lack of genetic variation within the banana population made it highly susceptible to a new strain of *Fusarium* that causes Panama disease. Genetic variability within a population has a direct impact on the virulence and ecology of certain pathogens because a highly variable gene pool allows them to adapt quicker to environmental change, thus increasing their potential to produce new virulent variants.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10327-018-00836-4) contains supplementary material, which is available to authorized users.

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Published online: 11 January 2019

The oomycete, *P. nicotianae*, first isolated by De Haan in 1896, is one of the most devastating oomycete plant pathogens in the world because its broad host range includes over 255 species across a wide diversity of climates around the world (Panabières et al. 2016). *P. nicotianae* was first reported in Japan in 1934 when it was isolated from *Agapanthus* seedlings with leaf blight by Takimoto and blight of lily by Tasugi and Kumazama (Asuyama 1934). At that time, *P. nicotianae* was reported under the name *P. parasitica*, which is now considered to be a synonym (Cline et al. 2008). Major outbreaks of *P. nicotianae* in Japan have caused root rot of strawberries (Matsuzaki 1988; Suzui et al. 1980). More recent reports of *P. nicotianae* in Japan have included a broad range of host plants such as poinsettia (Kanto et al. 2007), passion fruit (Horie 2007), citrus (Tashiro et al. 2002), asparagus (Yokota et al. 2013), Welsh onion (Takeuchi and Suzuki 2010), kalanchoe (Watanabe et al. 2007), New Zealand spinach (Takeuchi et al. 2004), garden pea (Takeuchi and Horie 2000) and *Limonium* (Nakamura and Matsuzaki 1994).

Population genetic studies of *P. nicotianae* have mainly focused on isolates from tobacco (Bonnet et al. 1994; Colas et al. 1998; Mammella et al. 2013). Recent analysis, using single nucleotide polymorphisms (SNPs) on mitochondrial and nuclear genes, grouped the isolates based on their host plants (Mammella et al. 2013). However, isolates from nurseries exhibit less association between the host plant and genetic grouping (Biasi et al. 2016). The absence of geographic structure for *P. nicotianae* revealed a recent expansion of a single diverse population (Bruberg et al. 2011).

Various types of genetic markers, including mitochondrial DNA (mtDNA), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single nucleotide polymorphism (SNP) and microsatellites, are widely used in the study of population genetics. Factors such as the level of variability or marker sensitivity, the nature of the marker (e.g., dominant or co-dominant, multilocus or single locus) and available equipment need to be considered when selecting the most suitable marker for population genetic analysis (Sunnucks 2000). On the basis of these factors, we opted to use microsatellites for this population genetics study.

Microsatellites, often also referred to as simple sequence repeats (SSRs), are tandemly repeating units of DNA with a repeat size of 1–6 bp, flanked by regions of non-repetitive unique DNA sequences. Microsatellites are very sensitive markers with a high level of variability within their repeat sequence, which means that they can be used to detect alleles at a locus. They usually have a high mutation rate because they are in a noncoding region. Moreover, inheritance of microsatellites alleles is Mendelian. All these advantages make microsatellites an excellent genetic marker for high-resolution population analysis (Selkoe and Toonen 2006).

Data on population structure can help us gain a better understanding of the genotypic diversity among and within a population. The genetic structure of the pathogen population can affect the genetic resistance of that pathogen. The more genetically diverse a population, the more likely that the population will survive in threatening environments (Charlesworth 2015). In this study, we needed to develop a reliable microsatellite marker to obtain a robust and comprehensive data set on population structure. Due to the importance of understanding population genetics for disease management strategies, the objectives of this study were to (1) develop microsatellite markers that are reliable for *P. nicotianae* population genetic analysis and (2) summarize the genetic diversity of *P. nicotianae* in Japan.

Materials and methods

Phytophthora nicotianae isolates

We evaluated 138 isolates of *P. nicotianae*: 125 isolates from 38 host plants across 15 prefectures in Japan, four isolates from Taiwan, two from the United States, and seven from Indonesia (Table 1). Some isolates were obtained from the culture collections of Gifu University, Japan Ministry of Agriculture, Forestry, and Fisheries (MAFF) and National Institute of Technology and Evaluation Japan (NITE) Biological Research Center (NBRC) and others were isolated for this study from infected pineapple and tobacco plants in Indonesia (Table 1). To investigate local population dynamics, we collected 23 isolates from kalanchoe fields in Gifu (2004–2009) and 16 from strawberry and asparagus fields in Saga (2012–2013).

Phytophthora nicotianae was isolated from infected plant tissues on selective NARM agar as previously described (Morita and Tojo 2007). The resultant mycelia were then identified by sequencing the internal transcribed spacer (ITS) region and the *cytochrome c oxidase 1* (*COX1*) gene (Robideau et al. 2011). The isolates were categorized into nine population groups based on their geographical origin: five populations from the largest main island Honshu (Chubu, Kansai, Kanto, Kyushu, Shikoku) and the southern islands of Japan and three populations from overseas (Taiwan, USA, and Indonesia).

Mating type determination

Isolate mating types were determined as previously described (Parkunan et al. 2010). Unknown mating types were paired with known A1 and A2 isolates (CH92ALS11 and CH93ANE1, respectively) on V8 agar, then incubated until a mating zone formed and antheridia and oogonia were observed.

Table 1 Isolates of *Phytophthora nicotianae* used in this study

Working number	Population	Isolates	Host plant	Geographical origin	Isolation year	Mating type
1	Chubu	MAFF 712194	Periwinkle (*Catharanthus roseus*)	Aichi, Japan	1997	A2
2	Chubu	GK10NI2SH	Periwinkle (*Catharanthus roseus*)	Gifu, Gifu, Japan	2010	A1
3	Chubu	GK 08NI8S2	Periwinkle (*Cattaranthus roseus*)	Gifu, Gifu, Japan	2008	A1
4	Chubu	GK10NI1SH	Periwinkle (*Catharanthus roseus*)	Gifu, Gifu, Japan	2010	A1
5	Chubu	OINB113SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	A2
6	Chubu	OINB153	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	A2
7	Chubu	OINB172	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	A2
8	Chubu	OINB171SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	A2
9	Chubu	OINB171	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	A2
10	Chubu	OINB153SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	A2
11	Chubu	OINB 113	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	A2
12	Chubu	OINB 161	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	A2
13	Chubu	OIOL0591R	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2005	A2
14	Chubu	OINO 451 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2005	A2
15	Chubu	OINO 451	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2005	A2
16	Chubu	OIOLO 0581 R	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2005	A2
17	Chubu	0705W21	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2007	A2
18	Chubu	0805WJ21	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2008	A1
19	Chubu	09E11294	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2009	A1
20	Chubu	09E321 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2009	A2
21	Chubu	09W422 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2009	A2
22	Chubu	09WCRS1-1	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2009	A2
23	Chubu	09W221 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2009	A2
24	Chubu	PHK6214 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	Unknown	A2
25	Chubu	GK 4-11	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	Unknown	A2
26	Chubu	PHKq-11	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	Unknown	A2
27	Chubu	PGS1 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	Unknown	A2
28	Chubu	MAFF 712342	China doll (*Radermachera sinica*)	Ise, Mie, Japan	Unknown	A2
29	Chubu	NBRC 30595	Strawberry (*Fragaria xananassa*)	Shizuoka, Japan	1979	nr
30	Chubu	MAFF 305926	Strawberry (*Fragaria xananassa*)	Shizuoka, Japan	Unknown	A2
31	Chubu	GF468	Strawberry (*Fragaria xananassa*)	Gifu, Japan	2003	A2
32	Chubu	GF524	Rose of Sharon (*Hibiscus syriacus*)	Ogaki, Gifu, Japan	2003	A2
33	Kansai	CH00POIN 2	Poinsettia (*Euphorbia pulcherrima*)	Hyogo, Japan	2000	A2
34	Kansai	CH00POIN3	Poinsettia (*Euphorbia pulcherrima*)	Hyogo, Japan	2000	A2
35	Kansai	MAFF 239554	Poinsettia (*Euphorbia pulcherrima*)	Hyogo, Japan	2003	A2
36	Kanto	CH08DAV11	*Euphorbia* sp.	Chiba, Japan	2008	A2
37	Kanto	C23	Indian mallow (*Abutilon* sp.)	Chiba, Japan	2007	A2
38	Kanto	C24	Indian mallow (*Abutilon* sp.)	Tateyama, Chiba, Japan	2007	A2
39	Kanto	MAFF305795	African violet (*Saintpaulia goetzeana*)	Tachikawa, Tokyo, Japan	1987	A2
40	Kanto	CH94AROE1	*Aloe vera*	Miyoshi, Chiba, Japan	1994	A2a
41	Kanto	CH94AROE3	*Aloe vera*	Miyoshi, Chiba, Japan	1994	A2[a]
42	Kanto	CH92ALS11	Peruvian lily (*Alstroemeria* sp.)	Kyonan, Chiba, Japan	1992	A2[a]
43	Kanto	CH92ALS21	Peruvian lily (*Alstroemeria* sp.)	Kyonan, Chiba, Japan	1992	A2[a]
44	Kanto	GUGC5631	Peruvian lily (*Alstroemeria* sp.)	Kyonan, Chiba, Japan	1992	A2[a]
45	Kanto	CH93ANE1	Spanish marigold (*Anemone coronaria*)	Kimitsu, Chiba, Japan	1993	A1[a]
46	Kanto	CH93ANE2	Spanish marigold (*Anemone coronaria*)	Kimitsu, Chiba, Japan	1993	A1[a]
47	Kanto	CH 90-4	Zebra plant (*Aphelandra squarrosa*)	Chiba, Chiba, Japan	1990	A2[a]
48	Kanto	CH90-9	Zebra plant (*Aphelandra squarrosa*)	Chiba, Japan	1990	A2[a]
49	Kanto	CH90-6	Zebra plant (*Aphelandra squarrosa*)	Chiba, Japan	1990	A2[a]

Table 1 (continued)

Working number	Population	Isolates	Host plant	Geographical origin	Isolation year	Mating type
50	Kanto	CH89-44	*Bougenvillea* sp.	Kyonan, Chiba, Japan	1989	A2[a]
51	Kanto	CH89-43	*Bougenvillia* sp.	Kyonan, Chiba, Japan	1989	A2[a]
52	Kanto	C38	*Brodiaea* sp.	Chiba, Japan	2007	A2
53	Kanto	MAFF 305796	Periwinkle (*Cathtaranthus roseus*)	Tokyo, Japan	1988	
54	Kanto	CH98Y1A	Yuzu (*Citrus junos*)	Futtsu, Chiba, Japan	1998	A1[a]
55	Kanto	CH98U1A	Tangerine (*Citrus unshiu*)	Futtsu, Chiba, Japan	1998	A1[a]
56	Kanto	MAFF 235436	*Daphne* sp.	Ibaraki, Tsukuba, Japan	1983	Nr
57	Kanto	CH95PHJ2	Winter daphne (*Daphne odora*)	Asahi, Chiba, Japan	1995	A2[a]
58	Kanto	CH95PHJ1	Winter daphne (*Daphne odora*)	Asahi, Chiba, Japan	1995	A2[a]
59	Kanto	CH87CWE1	*Dianthus* sp.	Wada, Chiba, Japan	1987	A2[a]
60	Kanto	CH87-51	*Dianthus* sp.	Chikura, Chiba, Japan	1987	A2[a]
61	Kanto	GUGC5562	*Dianthus* sp.	Chikura, Chiba, Japan	1987	A2[a]
62	Kanto	CH87KTK1	Carnation (*Dianthus caryophyllus*)	Tomiura, Chiba, Japan	1987	A2[a]
63	Kanto	CH87WG1	Carnation (*Dianthus caryophyllus*)	Wada, Chiba, Japan	1987	A2[a]
64	Kanto	CH87CWG1	Carnation (*Dianthus caryophyllus*)	Wada, Chiba, Japan	1987	A2[a]
65	Kanto	CH87-50	*Dianthus* sp.	Chiba, Japan	1987	A2[a]
66	Kanto	C15	*Echium fastuosum*	Tateyama, Chiba, Japan	2006	A2
67	Kanto	C58	*Gerbera* sp.	Chiba, Japan	2008	A2
68	Kanto	CH96HE1	English ivy (*Hedera helix*)	Kyonan, Chiba, Japan	1996	A2[a]
69	Kanto	CH97HE11	English ivy (*Hedera helix*)	Maruyama, Chiba, Japan	1997	A2[a]
70	Kanto	CH96HE2	English ivy (*Hedera helix*)	Kyonan, Chiba, Japan	1996	A2[a]
71	Kanto	C26	Lavender (*Lavandula angustifolia*)	Chiba, Japan	2007	A2
72	Kanto	CH99LK1	Lily (*Lilium hybrida*)	Kyonan, Chiba, Japan	1999	A2[a]
73	Kanto	CH91KK4	Easter lily (*Lilium longiflorum*)	Kyonan, Chiba, Japan	1991	A2[a]
74	Kanto	GUGC5567	Easter lily (*Lilium longiflorum*)	Kyonan, Chiba, Japan	1991	A2[a]
75	Kanto	GUGC5630	*Limonium* sp.	Maruyama, Chiba, Japan	1991	A2[a]
76	Kanto	GUGC5673	*Limonium* sp.	Maruyama, Chiba, Japan	1991	A2[a]
77	Kanto	CH91-33	*Limonium* sp.	Maruyama, Chiba, Japan	1991	A2[a]
78	Kanto	CH91-29	*Limonium* sp.	Maruyama, Chiba, Japan	1991	A2[a]
79	Kanto	CH92ORN21	*Ornithogallum* sp.	Futtsu, Chiba, Japan	1992	A2[a]
80	Kanto	CH92ORN11	*Ornithogallum* sp.	Futtsu, Chiba, Japan	1992	A2[a]
81	Kanto	CH93ORN4	*Ornithogallum* sp.	Tateyama, Chiba, Japan	1993	A2[a]
82	Kanto	GUGC5632	*Ornithogallum* sp.	Futtsu, Chiba, Japan	1992	A2[a]
83	Kanto	MAFF 712287	*Viola tricolor*	Saitama, Japan	2006	A1
84	Kanto	CH85PHP37	*Petroselinum crispum*	Maruyama, Chiba, Japan	1985	A2[a]
85	Kanto	CH85PHP61	*Petroselinum crispum*	Maruyama, Chiba, Japan	1985	A2[a]
86	Kanto	CH075STR81	Strawberry (*Fragaria xananassa*)	Chiba, Japan	2007	A2[a]
87	Kanto	CH91-1	*Strelitzia* sp.	Tateyama, Chiba, Japan	1991	A2[a]
88	Kanto	CH91-4	*Strelitzia* sp.	Tateyama, Chiba, Japan	1991	A2[a]
89	Kanto	CH91-3	*Strelitzia* sp.	Tateyama, Chiba, Japan	1991	A2[a]
90	Kanto	CH91-2	*Strelitzia* sp.	Tateyama, Chiba, Japan	1991	A2*
91	Kanto	GUGC5633	*Strelitzia* sp.	Chiba, Chiba, Japan	1991	A2*
92	Kanto	MAFF 305939	*Nicotiana rustica*	Kanagawa, Japan	Unknown	Nr
93	Kanto	CH89-39	*Vanda* sp.	Tateyama, Chiba, Japan	1989	A2[a]
94	Kanto	CH89-40	*Vanda* sp.	Tateyama, Chiba, Japan	1989	A2[a]
95	Kanto	CH99TK2	Lily (*Lilium hybrida*)	Chiba, Japan	1999	Nr
96	Kyushu	SG12ASP1-1	Asparagus (*Asparagus officinalis*)	Saga, Japan	2012	A2
97	Kyushu	SG12ASP1-2	Asparagus (*Asparagus officinalis*)	Saga, Japan	2012	A2
98	Kyushu	SG12ASP2-1	Asparagus (*Asparagus officinalis*)	Saga, Japan	2012	A2

Table 1 (continued)

Working number	Population	Isolates	Host plant	Geographical origin	Isolation year	Mating type
99	Kyushu	SG12ASP1-3	Asparagus (*Asparagus officinalis*)	Saga, Japan	2012	Nr
100	Kyushu	SG12ASP2-2	Asparagus (*Asparagus officinalis*)	Saga, Japan	2012	A2
101	Kyushu	SG13ASP1-2	Asparagus (*Asparagus officinalis*)	Saga, Japan	2013	A2
102	Kyushu	SG13ASP1-1	Asparagus (*Asparagus officinalis*)	Saga, Japan	2013	A2
103	Kyushu	SG13ASP1-3	Asparagus (*Asparagus officinalis*)	Saga, Japan	2013	A1
104	Kyushu	MAFF 237653	Strawberry (*Fragaria xananassa*)	Saga, Japan	1978	A2
105	Kyushu	MAFF 242197	Strawberry (*Fragaria xananassa*)	Saga, Japan	2004	A2
106	Kyushu	SGPC 0503	Strawberry (*Fragaria xananassa*)	Saga, Japan	Unknown	A2
107	Kyushu	SGPY 2101	Strawberry (*Fragaria xananassa*)	Saga, Japan	Unknown	A2
108	Kyushu	SGPC 0502	Strawberry (*Fragaria xananassa*)	Saga, Japan	Unknown	A2
109	Kyushu	SGPC 04118	Strawberry (*Fragaria xananassa*)	Saga, Japan	Unknown	A2
110	Kyushu	SGPC 0501	Strawberry (*Fragaria xananassa*)	Saga, Japan	Unknown	A2
111	Kyushu	SGHP0002	Strawberry (*Fragaria xananassa*)	Saga, Japan	Unknown	A2
112	Kyushu	MAFF 305940	*Nicotiana rustica*	Kagoshima, Japan	1977	A2
113	Kyushu	SE759	na	Saga, Japan		A2
114	Kyushu	F03	na	Fukuoka, Japan	2006	A1
115	Shikoku	MAFF 238154	Onion (*Allium cepa*)	Kochi, Japan	1999	A1
116	Shikoku	NBRC 33191	Scallion (*Allium fistulosum*)	Kochi, Japan	1999	A2[a]
117	Shikoku	NBRC 33190	Scallion (*Allium fistulosum*)	Kochi, Japan	1999	A2[a]
118	Shikoku	MAFF 238152	*Lilium* sp.	Kochi, Japan	1999	A2
119	Shikoku	NBRC 33193	*Lilium* sp.	Kochi, Japan	1999	A2[a]
120	Shikoku	NBRC 33192	Flame lily (*Gloriosa superba*)	Kochi, Japan	1999	A2
121	Southern Island	MAFF 305,797	*Dracaena* sp.	Hachijojima, Tokyo, Japan	1986	Nr
122	Southern Island	MAFF 305591	Papaya (*Carica papaya*)	Ogasawara, Tokyo, Japan	1986	A2
123	Southern Island	MAFF 305799	Passion fruit (*Passiflora edulis*)	Hachijojima, Tokyo, Japan	1983	A2
124	Southern Island	MAFF 305978	Passion fruit (*Passiflora edulis*)	Ogasawara, Tokyo, Japan	1988	A2
125	Southern Island	MAFF 305590	Tomato (*Solanum lycopersicum*)	Ogasawara, Tokyo, Japan	1986	Nr
126	Taiwan	NBRC 31425	Onion (*Allium cepa*)	Taiwan	1984	A1[a]
127	Taiwan	NBRC 31423	Pineapple (*Annanas comosus*)	Taiwan	1984	A1[a]
128	Taiwan	NBRC 31419	Papaya (*Carica papaya*)	Taiwan	1984	A2[a]
129	Taiwan	NBRC 31416	Tomato (*Solanum lycopersicum*)	Taiwan	1984	A2[a]
130	Indonesia	TBC GTS	Tobacco (*Nicotiana rustica*)	Central Java, Indonesia	2016	A1
131	Indonesia	AA 129D 2	Pineapple (*Annanas comosus*)	Lampung, Indonesia	2016	A1
132	Indonesia	AA 71A S1	Pineapple (*Annanas comosus*)	Lampung, Indonesia	2016	A2
133	Indonesia	AA 114K HS 2	Pineapple (*Annanas comosus*)	Lampung, Indonesia	2016	A1
134	Indonesia	AA 71A 2	Pineapple (*Annanas comosus*)	Lampung, Indonesia	2016	A2
135	Indonesia	AA 36G	Pineapple (*Annanas comosus*)	Lampung, Indonesia	2016	A1
136	Indonesia	AA 71A 3	Pineapple (*Annanas comosus*)	Lampung, Indonesia	2016	A1
137	USA	CBS 535.92	Soil	USA		A1[a]
138	USA	CBS 534.92	Soil	USA		A2[a]

Nr no mating reaction, *Unknown* information not available

[a]Mating type data were provided on the origin of the isolates

Microsatellite marker development

The complete genome sequence of *P. nicotianae* was screened for the microsatellite motifs using Tandem Repeat Finder (Benson 1999). The alignment parameters for Tandem Repeat Finder were 2, 3 and 5, and only those repeats with a minimum score of 80 and a maximum period size of 6 were reported. The microsatellites were selected on the basis of a minimum of three repeats for trinucleotides and tetranucleotides. Primers flanking the identified loci

were designed, and their specificity was confirmed using Primer BLAST (Ye et al. 2012). All primers were designed using the following criteria: Tm of 55–65°C (optimum at 58°C), product size of 150–250 bp (optimum at 200 bp), GC content 45–60% (optimum at 50%) and primer size of 18–25 bp (optimum at 20 bp).

All primers were analyzed for hairpin and dimer potential using NetPrimer (http://www.premierbiosoft.com/NetPrimer/AnalyzePrimer.jsp) to select the best primer pairs. These selected primer pairs were then analyzed against the whole genome sequence of *P. nicotianae* by in silico PCR using the Web-based program insilico.ehu.eus (San Millán et al. 2013). Amplified fragments were cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and then sequenced to characterize their microsatellite motifs. More than 12 *E. coli* recombinants were selected by colony PCR and purified using the ExoSAP-IT kit, following the manufacturer's instructions (Affimetrix, Santa Clara, CA, USA). The purified PCR product was sequenced using the M13M4 primer for amplification by the BigDye Sequence Terminator Kit (Applied Biosystems, Foster City, CA, USA) on an ABI3500 automated sequencer (Applied Biosystems).

Microsatellite genotyping

The developed polymorphic loci were used to analyze all 138 isolates. The primers were labeled at the 5′ end separately with the fluorescent dye FAM (6-carboxy-fluorescein) or HEX (4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein) (Lees et al. 2006).

The total genomic DNA was extracted using PrepMan Ultra Reagent (Applied Biosystem) and amplified using all selected primers under the following conditions: 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 7 min. Reactions were performed in a total volume of 25 µl containing 2 µl of 1 ng DNA, 2.5 µl of 10× PCR Buffer (plus magnesium, Takara Bio, Otsu, Shiga, Japan), 2.5 µl of 4 mg/ml BSA, 2.5 µl of 10 mM primer (forward and reverse), 2 µl of 2.5 mM dNTP mix (Takara Bio), 0.1 µl rTaq polymerase (Takara Bio), and 10.9 µl ddH_2O. PCR amplification products were separated in 2% agarose gels in 0.5 × Tris-acetate-EDTA buffer, stained with GelRed (Biotium, Fremont, CA, USA) and visualized under UV light.

After confirmation of the PCR product, fragments were analyzed on an ABI3100 or ABI3130 Genetic Analyzer (Applied Biosystem) using the LIZ 250 DNA ladder as a marker. The electropherogram was scored manually.

Population structure analysis

In a cluster analysis of the population structure, the probability of genotypes being distributed into *K* number of clusters was estimated using STRUCTURE v. 2.3.4. (Falush et al. 2003, 2007; Hubisz et al. 2009; Pritchard et al. 2000) with an admixture model without prior population information and 200,000 Markov chain Monte Carlo (MCMC) iterations. Eight independent runs were performed for each $K = 1–20$. The optimal number of *K* was selected by STRUCTURE HARVESTER (Earl and Von Holdt 2012) and matched from an independent run by CLUMPP (Jakobsson and Rosenberg 2007). The result was then finally visualized using DISTRUCT (Rosenberg 2004). The distant matrix created by GenAlex 5.6.3. (Peakall and Smouse 2006, 2012) was used for phylogenetic analysis using a neighbor-joining algorithm in MEGA 6.0 (Tamura et al. 2013). The mating pattern within the population was statistically analyzed using an analysis of molecular variance (AMOVA) in GenAlex 5.6.3 (Peakall and Smouse 2006, 2012).

Results

Development of microsatellite markers

The entire genome sequence was screened using Tandem Repeat Finder, and 12 primer sets were selected that could specifically amplify 12 microsatellite loci of *P. nicotianae*. Those primer sets were then tested on three isolates (GUCC 5620, 5621 and 5623), and the loci that had multiple alleles were selected for study (Table 2). The selection of microsatellite markers established six novel polymorphic microsatellite loci. Six of 12 selected primer sets were suitable for population structure analysis because they were amplified in all isolates, diploid, and highly polymorphic.

In total, 39 alleles were detected from six loci, ranging from four (TAA) to 11 (GTA) alleles per locus, with an average of 6.5 (Table 3) and maximum of 11 at locus GTA. This locus was also the most informative, as it had the highest Shannon's Information Index ($I = 1.838$). Two of six alleles had significantly higher observed heterozygosity, while the rest were significantly lower. All of the loci significantly differed from Hardy–Weinberg equilibrium (HWE).

Mating type distribution

From 138 isolates, 21 isolates were identified as mating type A1, 95 as A2, and 22 isolates had no reaction to either the A1 or A2 mating type. Both A1 and A2 mating types were found on one kalanchoe farm in Gifu (Japan), one asparagus farm (Saga), one onion field (Kochi), and one pineapple field (Lampung, Indonesia). On the kalanchoe and asparagus farms, the A1 and A2 mating types were isolated in different years, while both mating types were isolated the same year in the Indonesian pineapple field (Table 1).

Table 2 Novel microsatellite markers of *Phytophthora nicotianae* developed in this study

Locus	Repeat motif	Primer sequence	Annealing temperature (°C)	Fluorescent label	Alleles	
					N	Size
AA-TTA	TTA	F: CGTGAGGCAGATGCTGTCAA R: TGGGTTTCAGCCCTTCAACT	60	FAM	4	263–287
AA-AAC	AAC	F: GAGTTCTACATCCCGGTTCCA R: GCTTATAGTGGTGCAAGCGTC	60	FAM	10	193–220
AA-GCT	GCT	F: CTGGACATGCTCAGGGTGTT R: GACTGGATGGATCCGGCTTG	60	FAM	5	177–189
AA-CAG	CAG	F: ACGACCCATTCGCTGTTCAA R: TTTCCGTTGTTTGTGGGTGC	60	HEX	4	234–246
AA-TAA	TAA	F: TCTACGTCAGGGCGGTTTTT R: GAAATGTGTGGGTCAGTCGC	60	HEX	4	170–179
AA-GAA	GAA	F: GTGTCTTCACTGTCACCGGCAGTAGAA R: GTGTCTTCGGTTGGTCCAAACCTCTCC	60	HEX	5	282–294

Table 3 Microsatellite characteristics

Locus	Na	Ne	*I*	H_o	H_e	*p*	F_{ST}
AA-GAA	5	2.401	1.068	0.779	0.584	0	0.072
AA-GTA	11	5.167	1.838	0.717	0.806	0	0.199
AA-AAC	7	3.798	1.484	0.649	0.737	0	0.18
AA-CAG	7	1.865	0.837	0.462	0.464	0	0.061
AA-TTA	5	3.691	1.357	0.752	0.729	0.002	0.119
AA-TAA	4	1.246	0.442	0.137	0.197	0	0.111

Na number of alleles, *Ne* number of expected alleles, *I* Shannon's Information Index, H_o observed heterozygosity, H_e expected heterozygosity, *p p* value for Hardy–Weinberg's equilibrium, F_{ST} Fixation Index

Phylogenetic analysis

The phylogenetic tree constructed with the neighbor joining algorithm revealed five major clades (Fig. 1). The isolates collected from the Kanto area were scattered in all clades in the phylogenetic tree as well as the isolates collected from Taiwan, Shikoku, and the southern islands. Isolates from the same geographic origin but different host species were found to be distantly related as were isolates from the same host species but different geographic origins. However, several isolates had the same genotype as other isolates collected from the same host and same geographic origin: three isolates (working number 76–78) from *Limonium* sp. in Chiba (clade 1), two isolates (No. 5 and 11) from *Kalanchoe* sp. in Gifu (Clade 2), two isolates (No. 9 and 12) also collected from *Kalanchoe* sp. in Gifu (Clade 3), two isolates (No. 9 and 100) from asparagus in Saga (Clade 3), four isolates (No. 61–64) from carnation in Chiba (Clade 4), and two isolates (No. 87 and 88) from bird of paradise flower.

The isolates collected from the same host and geographic origin in different years were observed to have different genotypes. The isolates from kalanchoe in Gifu in 2004 were grouped in clades 2 and 3 (No. 10, 9, 12; and 5–8, 11, respectively), while the isolates collected in 2007 (No. 17) were found in the Clade 1. Isolates collected from *Ornithogallum* sp. in Chiba in 1992 (No. 80 and 82) were grouped in Clade 4, while the isolates collected in 1993 (No. 81) clustered in Clade 5. Interestingly, the isolate (No. 81) from *Ornithogallum* sp. had the same genotype as a parsley isolate (No. 85), although it was collected from a different host and geographic origin.

Population structure analysis

Cluster analysis revealed that the optimal number of genotypic clusters represented within the data was $K=5$ and that all isolates had all clusters at different proportions. Furthermore, the populations consisted of highly admixed individuals (Fig. 2a). However, several genotypic clusters were found to be predominant in one area but minor in another. In Fig. 2, the blue cluster was prevalent in populations from Kyushu, Taiwan, and Indonesia. A fourth cluster (green) was identified in populations from Japan, Taiwan, and Indonesia but rarely in those from the United States. The yellow cluster was predominant in the southern islands and U.S. populations (Fig. 2b).

The analysis of molecular variance (AMOVA) of microsatellite genotype data showed that isolates have low diversity

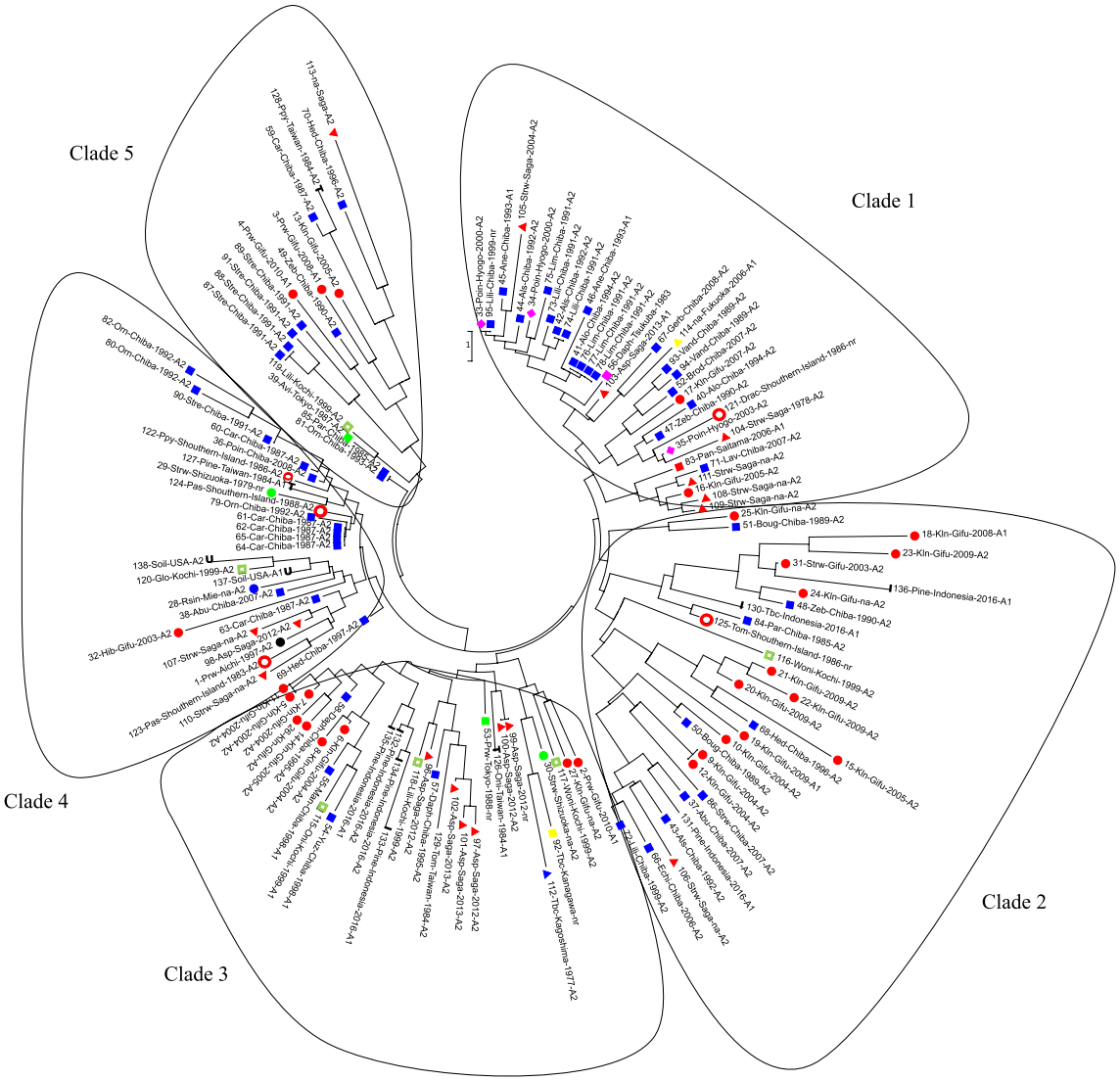

Fig. 1 Phylogenetics analysis of *P. nicotianae* populations. Each branch labelled with: isolates number-host plantsgeographic origin-year of isolation-mating type. Host abbreviations—Poin: poinsettia; Lili: *Lilium* sp.; Als: *Alstroemeria* sp.; Ane: anemone; Alo: *Aloe vera*; Lim: limonium; Asp: asparagus; Gerb: *Gerbera*; Vand: *Vanda* sp.; Brod: *Brodiaea* sp.; Kln: kalanchoe; Strw: strawberry; Drac: *Dracaena* sp.; Lav: lavender; Boug: *Bougenvillea* sp.; Pine: pineapple; Tbc: tobacco; Abu: *Abutilon*; Echi: *Echium*; Prw: *periwinkle*; Woni: Whelsh onion; Oni: onion; Daph: daphne; Pas: passion fruit; Car: carnation; Hib: hibiscus; Rsin: *Radermachera sinica*; Orn: *Ornithogallum*; Zeb: zebra plant; Hed: *Hedera* (English ivy)

among the populations (3%) but high diversity among individuals within a population (Table 4). The low Fixation Index (F_{ST}) of 0.033 with a p value of 0.082 meant that there was no significant difference between populations and a high possibility of gene flow between the populations and limited contribution of geographical origin to the genetic variance of *P. nicotianae* populations in Japan.

Discussion

Previous studies on the population genetics of *P. nicotianae* have been based on mitochondrial and nuclear DNA (Colas et al. 1998; Mammella et al. 2013). Importantly, these studies used isolates from Australia and the United

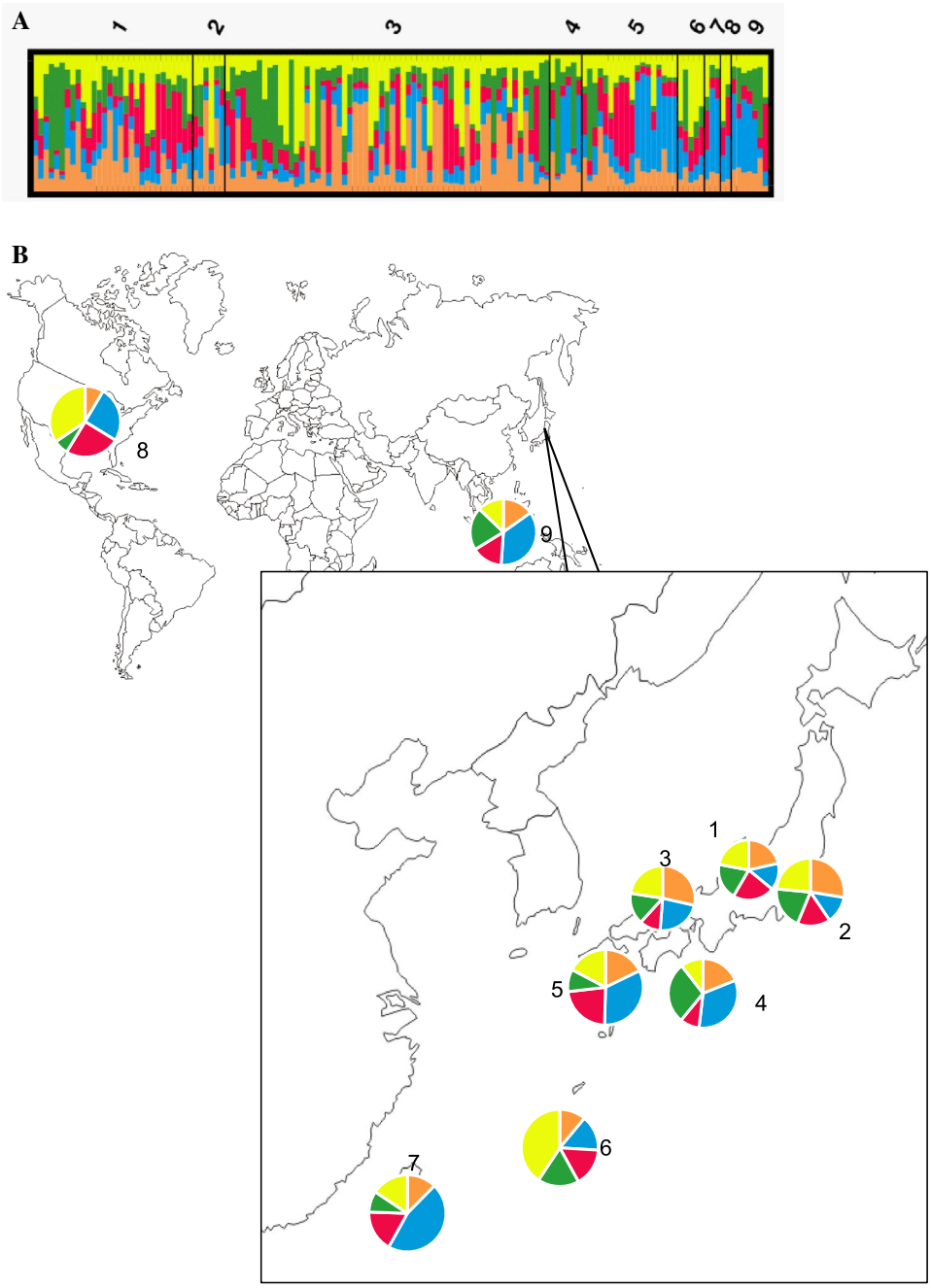

Fig. 2 Cluster analysis of *Phytophthora nicotianae* using STRUCTURE v. 2.3.4 (**a**). Genotypic clustering in each population (1: Chubu; 2: Kanto; 3: Kansai; 4: Shikoku; 5: Kyushu; 6: southern islands; 7: Taiwan; 8: USA; 9: Indonesia). **b** Proportions of the genotypic clusters in each population

Table 4 Summary of analysis of molecular variance (AMOVA) of *Phytophthora nicotianae* populations used in this study

Source	*df*	SS	MS	Est. var.	%	$F_{statistic}$	*p* value
Among populations	8	28.014	3.502	0.064	3	$F_{ST}=0.033$	0.087
Within populations	267	500.812	1.876	1.876	97		
Total	275	528.826		1.940	100		

df degree of freedom, *SS* sum of squares, *MS* mean square, *Est. var.* estimated variance, % percentage of variance, F_{ST} Fixation Index, *Pops* populations

States, so little was known about *P. nicotianae* populations from Japan. To provide a better understanding of how the pathogen is likely to emerge at a more local level in Japan, here we developed novel microsatellite markers to amplify six loci from 138 isolates from six regions in Japan and 13 isolates from overseas for comparison. A high level of polymorphism was revealed, ranging from 4 (AA-CAG) to 11 (AA-GTA) alleles per locus (Table 3). The 39 alleles amplified from six microsatellite loci is much higher than reported for *P. infestans* (Montarry et al. 2010) and *P. sojae* (Wu et al. 2017) perhaps due to the broader host range of *P. nicotianae*. *P. capsici*, which also has a wide host range, was reported to have 5–14 alleles per locus (Meitz et al. 2010), while *P. alni*, with a narrow host range, has as few as 2–3 per locus (Aguayo et al. 2010).

The pathogen isolated from kalanchoe in Gifu was scattered across several clades of the phylogenetic tree (Fig. 1). These isolates were collected from the same farm. In this case, the different year of isolation was a significant factor. Isolates from 2004, 2008, and 2009 were grouped into clades 2 and 3 on the phylogenetic tree, whilst the isolates from 2005 to 2007 occupied Clade 1. Novel genetic variance found in the different years of isolation could have been introduced via plant materials (such as potting mixture, seedlings, or irrigation water) because all the isolates from the previous year were type A2, thus preventing sexual recombination.

By contrast, isolates from the Saga Prefecture tended to group according to mating type and host, rather than year of isolation. Type A2 isolates from asparagus were grouped in Clade 3, even though they were collected during a different year, while type A1 was in the Clade 1. The isolates from pineapple in Indonesia were also grouped into a single clade (Clade 3) and differed from the isolates from pineapple in Taiwan (Clade 4). These results show that the sources of infection are local and specific to those host plants.

The clustering and statistical analysis revealed that *P. nicotianae* in Japan had high variation among individuals and a lack of geographical structure. Cluster analysis using STRUCTURE showed that the *P. nicotianae* in Japan is highly admixed in all the isolates because there was less than 80% similarity within any one genetic cluster. This admixture could benefit the pathogen by increasing the degree of genetic variation within the population, thus raising the likelihood that novel genotypes with new combinations of traits will arise through natural selection and that deleterious mutations caused by inbreeding will be masked (Verhoeven et al. 2011). This condition is likely to be due to the choice of host plants used in this study, the majority of which were ornamental. Isolates from ornamental species are more likely to exhibit high genetic variation due to the admixtures of diverse genotypes, resulting from the trading of infected plant material between nurseries in different countries (Biasi et al. 2016).

Inconsistency between genotypic clusters and geographical origins are common in demographic analyses of *Phytophthora* species. Previous studies on *P. nicotianae* isolated on citrus (Biasi et al. 2016), *P. plurivora* (Schoebel et al. 2014), and *P. colocasiae* (Nath et al. 2013) also showed moderate to high genetic diversity without any clear relationship with the geographical origin. In the present study, the high number of genotypic clusters in a population was found to be linear to the percentage of variance among the individuals of the population. Because the AMOVA tests confirmed that variance was high within the population (97%) and low among the population (3%), while the number of genotypic clusters suggested by STRUCTURE HARVESTER was relatively high ($\Delta K = 5$). The low number of F_{ST} value (0.033) and the associated p value of 0.08 revealed that there was no significant genetic differentiation among populations. The undifferentiated population indicates the possibility of sharing genetic materials between the populations (Ma et al. 2015), which could explain why there was no strong geographical structuring in the Japanese populations of *P. nicotianae*.

The lack of strong geographical structure in the *P. nicotianae* populations in Japan could be evidence that isolates have migrated via human activities. Since *P. nicotianae* is soil- and water-borne and can survive in its chlamydospore state for a long time, it could be transported via agricultural products or watercourses. Both the phylogenetic analysis and population structure results agree with a previous study in which it was hypothesized that *P. nicotianae* has been spread worldwide via plant material and subsequent progressive lineage diversion (Mammella et al. 2013). The pathogen was likely to respond rapidly to natural selection imposed by newly introduced host resistance genes or fungicides (Nath et al. 2013) Moreover, the ability of *P. nicotianae* to reproduce both sexually and asexually will enable the pathogen to be more genetically diverse. While this study has not identified the original source of *P. nicotianae* in Japan, it has provided a better understanding of *P. nicotianae* gene flow and of its evolutionary potential in Japan. Further studies should include isolates from nearby countries and improved sampling proportions to determine the route(s) of migration by *P. nicotianae*.

Acknowledgements The authors acknowledge Mr. Seiji Uematsu, Dr. Hideki Watanabe, Mr. Minoru Inada, Dr. Yuji Kajitani for providing *P. nicotianae* isolates used in this study.

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