Population Structure Analyses of Plant Pathogenic Oomycetes Using Microsatellite Markers

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（植物病原性卵菌類のマイクロサテライトマーカーによる個体群構造解析）

2018

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GENERAL INTRODUCTION

The biology of oomycetes

The oomycetes were also referred as “water mold” is a member of kingdom stramenopiles. This group include some devastating plant pathogen that cause seedling blight, damping- off, root rots, foliar blight, and downy mildews (Grünwald and Fry 2010). In the early taxonomic era, the oomycetes were classified as lower fungi due to its growing habit, spores producing for reproduction, nutrition intake by absorption, and similar ecological niche. However, the use of molecular data as sources of classification, revealed that the oomycetes were evolutionary closer to brown algae and diatoms rather than to a true fungi (Judelson 2012). Hence, recent classification were group the oomycetes on the straminopila kingdom along with brown algae and diatoms. It also join the Chromalveolata super kingdom linked with the ancestral endosymbiosis of pothosynthetic algae (Keeling 2009).

The relationship between major orders belonging to the oomycetes were constructed on the phylogenetic three using 28s ribosomal RNA sequences (Fig. 1). The oomycetes are primarily pathogen for animal, plant, or fungi. In the order Saprolegniales, *Aphanomycetes* causes root rot of a wide range hosts. In the order *Peronosporales,* several genera cause serious foliar diseases known as downy mildew, *Peronospora*, *Sclerospora*, *Plasmospora*, and *Brenia* (Link, Powelson, and Johnson 2012). The family *Pythiaceae* initially consists of two genera, *Phytophthora* and *Pythium*. These two genera were distinguish based on the shape of sporangia, the existence of sporangiophore, zoospore

differentiation, and formation of appressorium (Tewoldemedhin et al. 2011). However, in 2010 the new genus were described as *Phytopythium* (Bala et al. 2010), as it has globose *Phytophthora*-like sporangia and vesicle mediated *Pythium*-like zoospore discharge. The member of this genera were previously classified as *Pythium* clade K including *Py. sindhum*, *Py. litorale, Py. vexans, Py. Ostraccodes* and *Py. helicoides*.

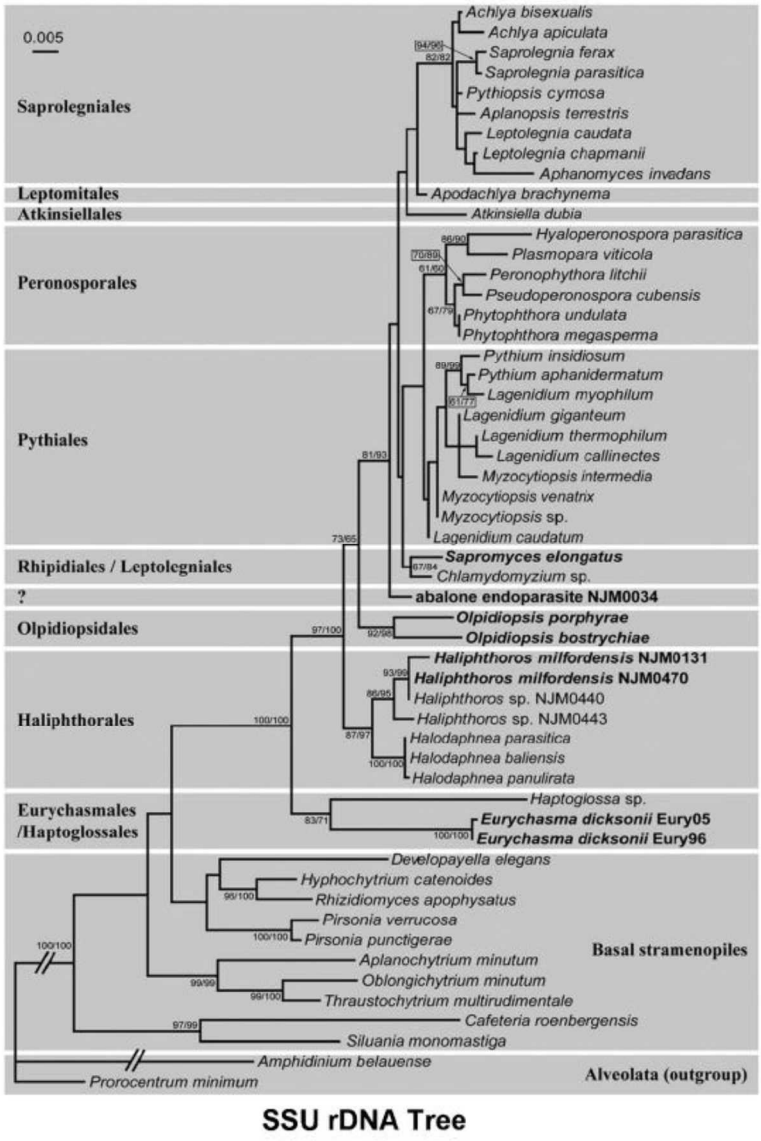

Figure 1. Phylogenetic relationship of taxa in the oomycete class and representatives chromealveolates (Beakes and Sekimoto 2009).

Sources of genetic variances in oomycetes

One of the key to the success of plant pathogenic oomycetes is their ability to adapt to overcome host resistance and jump to new host (Derevnina et al. 2016). This due to their ability to reproduce both sexually and asexually, making them able to gain both allelic diversity (sexual and interspecific hybridization) and to rapidly proliferate, multiplying the size of the population through asexual/ clonal reproduction (McDonald and Linde 2002).

Due to the clonal reproduction being predominant in oomycetes populations, mutations become one of the primary sources of genetic variation. The member of clonal lineage are descended from single individual with any variation arise within a lineage were caused by mutation. Some traits in the *Phytophthora* species were detected as caused by mutation, such as virulence in *P. infestans* and *P. sojae* (Drenth A. et al. 1993; Drenth et al. 1996). There was also evidences in fungicide resistance and genetic mutation in *Phytophthora* spp. Some notable cases are the metalaxyl resistance in *P. infestans* (Koh et al. 1994), carboxylic acid amide (CAA) resistance in *P. melonis* (L. Chen et al. 2012), pyrimorph in *P. capsica* (Pang et al. 2013), and phenylamide fungicide in *P. infestans* (Gisi and Cohen 1996).

In some species, diversity caused by mitotic recombination were detected. This variation did not generate new variation but can reveal the recessive variation that previously hidden as heterozygous (Goodwin 1997). The events were found in EC-1 lineage of *P. infestans* (Forbes et al. 1997), population of *P. ramorum* in North America and Europe (Ivors et al. 2004), and population of *P. cinnamomi* in Australia (Dobrowolski et al. 2003).

Oomycetes also exhibit variations of ploidy either triploids or polyploids. Polyploidy has been proved to buffer mutational changes by masking deleterious allele. In *P. infestans*, the higher ploidy levels were caused by adaptation to the cooler environment as it exist as tetraploid in Mexico, the species center of origin (Sansome 1977). The recent study revealed that inter-species hybridization as sources of polyploidy in *Phytophthora* spp. indicated a clear expansion of host range (Bertier et al. 2013).

Studies on population genetics of oomycetes

Advancement in molecular genetics was lead to population dynamics study on molecular level. Choosing molecular marker for population genetic analysis is a crucial task. These markers have to be neutral, polymorphic, as well as reproduceable to provide insight at the right evolutionary scale (Grünwald and Goss 2011). Several molecular markers were used on the past and recent studies for population genetic analysis of *Oomycetes.* Microsatellites can provide insight into recent divergence, while mitochondrial, nuclear, or other sequence loci provide more distant evolutionary history in slower mutation rates (Grünwald et al. 2017).

Study on population genetics of *P. sojae* using virulence and RFLP markers (Förster, Tyler, and Coffey 1994) revealed that the pathogen has low levels of genotypic the USA population. The study then continued with addition of Australian population (Drenth et al. 1996) which revealed a lower genotypic diversity of Australian population than USA population. The population genetics study on a global scale revealed that China could be the origin of *P. sojae* as where soybeans originated, because the existence of some resistance host (Huang et al. 2016; X. ling Wu et al. 2011).

The center of origin from pathogen can be understand by population genetic analysis as they usually has some criteria, such as the occurrence of widespread resistance in indigenous plants, the existence of more or less equal A1 and A2 mating types ratio, the occurrence of high level of genetic diversity, and little or no disturbance by man (Drenth and Goodwin 1999).

Species introduction: *Phytophthora nicotianae*

*Phytophthora nicotian*ae was first isolated by De Haan in 1896 from tobacco in Indonesia and is considered to be one of the most devastating oomycete plant pathogens in the world due to its broad host range of over 255 plant genera in 90 families. Its habitat ranging from herbaceous to woody plants in tropical, subtropical, as well as temperate region (Panabieres et al., 2016). This pathogen causes 100% yield loss in tobacco (Erwin and Ribeiro, 1996) and caused major loss in citrus, and tomato industries in Australia. As soil borne pathogen, *P. nicotianae* is indigenous in the natural environment such as mountainous area (Vannini, et al., 2009), forest soil (Maseko, et al., 2001), and irrigation water (Hong and Moorman, 2005).

Phytophthora nicotianae is a heterothallic species and forms aplerotic oospores from amphygenous antheridia and septate oogonia. The oospore size ranges from 23 to 38 μm in diameter. It produce non caducous sporangia with spherical, ovoid, and ellipsoid shape with size range from 56.0 × 35.0 to 33.3 × 24.5 μm. It also has 1 to 3 sharp papillae on each sporangium with a diameter of 25.4- 40.3 μm. The mycelia are arachnoid with hyphal swelling and form intercalary or terminal chlamydospores (Erwin and Riberio, 1993; Santos, 2016). The colony morphology is variable from rosette, stellate, and lanose

(Hall, 1993). The optimum temperature for mycelial growth are range between 24 and 32ºC and no grows above 36ºC.

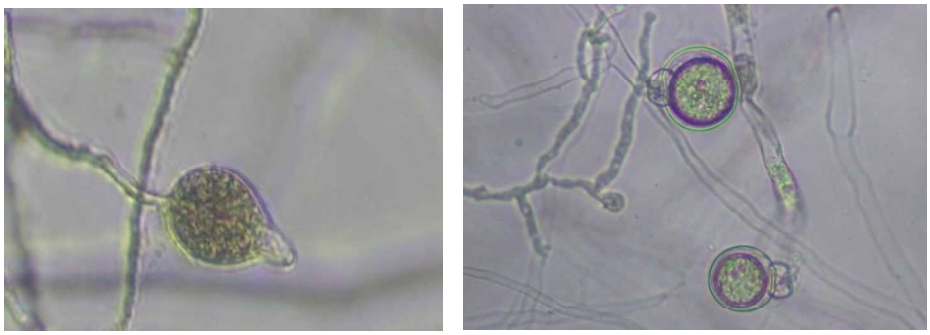

Figure 2. The morphological characteristics of *Phytophthora nicotianae*.

The chlamydospore of *P. nicotianae* is a survival resting structure as long as four to six years make the pathogen hard to get rid of. Once the environment is suitable for the pathogen to grow, the chlamydospores germinate to produce germ tube that infects plant or produce a sporangium. The sporangia can either germinate directly or release motile zoospores which able to migrate to more favorable condition (Fig. 2) (Gallup, et al., 2006; Meng, et al., 2014).

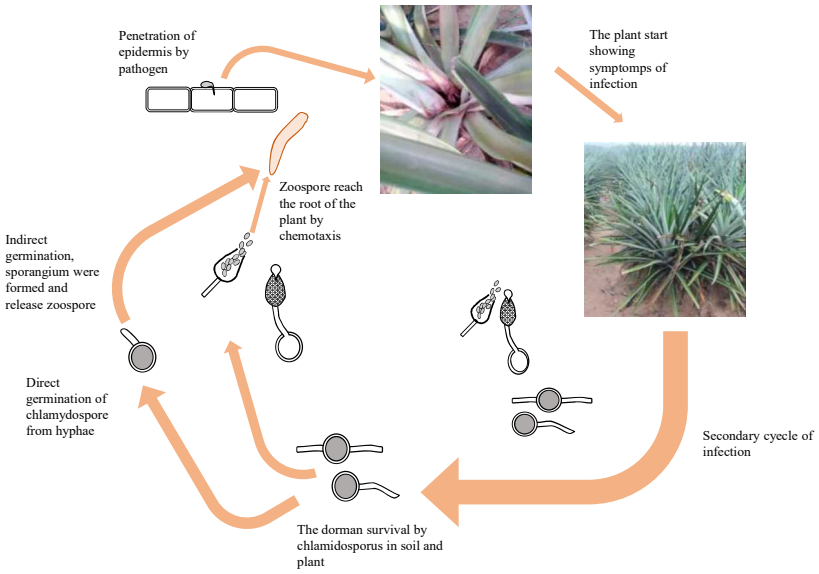

Figure 3. Life cycle of *Phytophthora nicotianae* in pineapple

Although *P. nicotianae* was first isolated in Indonesia, the report of the pathogen in Indonesia is limited. Some studies of the pathogen in Indonesia were restricted to the isolation and identification from several host plants such as coconut (Blaha et al., 1994) and Citrus (Marpaung et al., 2010; Henuk et al., 2017),

Species introduction: *Phytopythium helicoides*

Phytopythium helicoides was first isolated on 1930 by Drechsler from dahlia in USA. After that, there has been few report until 1996 it was first isolated on the miniature rose in Gifu, Japan in 1996 (Kageyama et al. 2002), since then it has infected others agricultural important plants such as kalanchoe, kiwi, and strawberry (Watanabe et al. 2007; Ishiguro et al. 2014). In other country, this high temperature pathogen is recently causing root rot and stem rot on economically important plants such as bell peper and

pistachio in the USA (Chellemi et al. 2000), citrus mandarin and kiwi fruit in china (X. R. Chen et al. 2016; Wang et al. 2015), and rose in Korea (Han et al 2010).

Previous study on genetic diversity of *P. helicoides* revealed the high variability of the rDNA ITS region within a single isolate, suggesting that this pathogen undergo cross-breeding among isolates within the population (Kageyama et al. 2007). Inspite of a homothallic species, asexual strain of *Ph. helicoides* also isolated from diseased plant. The asexual strain has no oospores but able to produce abundant sporangia as an effective survival structure on the green house where the humidity and temperature is maintained throughout the year (Kageyama et al. 2003). The evidence of high variability among isolates of *P. helicoides* may lead to a probability of the emergence of new strain on *P. helicoides* in the future.

Fig 4. Morphological characteristics of *Phytopythium helicoides* (a) globose sporangium with papilla; (b) interna proliferation; (c) aplerotic smooth sporangia.

CHAPTER I

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

In recent years, advancements in transportation technology have made global trading easier. The resultant global redistribution of species by human activities has included both the introduction of beneficial species to new environments but also the introduction of their associated pathogens. Nowadays, most crops are in the process of rapid biotic homogenization, potentially leading to significant reductions in the genetic variability of the principal crops of many important agricultural nations within the next few decades (Bebber, Holmes, and Gurr 2014). This poses a threat to crop species because if a new virulent variant of a pathogen evolves, it could have the potential to spread rapidly through the host plants, resulting in high losses and poor yields. The near extinction of the Gros Michel banana in the 1950's is a good example: the lack of genetic variation within the banana population made it highly susceptible to a new strain of *Fusarium* called the Panama disease. Genetic variability within a population has a direct impact on the virulence and ecology of certain pathogens because the possession of a highly variable gene pool allows them to adapt more quickly to environmental change and thus increases their potential to produce new virulent variants.

The Oomycete, *P. nicotian*ae, was first isolated by De Haan in 1896 and is considered to be one of the most devastating oomycete plant pathogens in the world due

to its broad host range of over 255 species from all over the world, across a wide diversity of climates (Panabières et al. 2016). The earliest reports of *P. nicotianae* in Japan were recorded in 1934 in which it was isolated from *Agapanthus* seedlings with leaf blight by Takimoto and blight of lily by Tasugi and Kumazama (Asuyama 1935). At that time, *P. nicotianae* was reported under the name *P. parassitica,* which is now considered to be a synonym (Cline, Farr, and Rossman 2007). Major outbreaks of *P. nicotianae* in Japan have caused root rot of strawberries (Suzui et al. 1980; Matsuzaki 1988). More recent reports of *P. nicotianae* in Japan have included a broad range of host plants such as poinsettia (Kanto, Uematsu, and Aino 2007), passion fruit (Horie 2007), citrus (Tashiro et al. 2002), asparagus (Yokota et al. 2013), Welsh onion (T. Takeuchi and Suzuki 2009), kalanchoe (Watanabe et al. 2007), New Zealand spinach (J. Takeuchi, Horie, and Eimori 2004), garden pea (J. Takeuchi and Horie 2000) and *Limonium* (Nakamura and Matsuzaki 1994).

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

There are several genetic markers that are widely used in the study of population genetics. These include markers for mitochondrial DNA (mtDNA), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single nucleotide polymorphism (SNP) and microsatellites. Several factors need to be

considered when selecting the most suitable marker for population genetic analysis, including: the level of variability or marker sensitivity; the nature of the information, whether it is dominant or co-dominant, multilocus or single locus; and the available laboratory equipment (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 genetic loci. They usually have a high mutation rate because they are in a non-coding region. Moreover, microsatellites alleles obey the Mendelian inheritance law. All these advantages make microsatellites a most suitable genetic marker for high resolution population analysis (Selkoe and Toonen 2006).

Data on a population's structure can help us to gain a better understanding of the genotypic diversity among and within that population. The genetic structure of a pathogen's population can affect the genetic resistance of that pathogen. The more genetically diverse a population, the more likely that the population will survive under threatening environmental conditions (Charlesworth 2015). In this study, it was necessary to develop a reliable microsatellite marker in order to gain a robust and comprehensive population structure data set. Due to the importance of understanding population genetics for disease management strategies, the objectives of this study were (i) to develop microsatellite markers that are reliable for *P. nicotianae* population genetic analysis and (ii) to review the genetic diversity of *P. nicotianae* in Japan.

Materials and methods

Phytophthora nicotianae isolates

In total we evaluated 138 isolates of *P. nicotianae*: 125 isolates from 38 host plants collected across 15 prefectures in Japan, four isolates from Taiwan, two from the USA, and seven isolates from Indonesia (Table 1). Some isolates were obtained from the culture collections of Gifu University, MAFF and NBRC, and others were purposely isolated for this study from infected pineapple and tobacco plants in Indonesia (Table 1). To investigate local population dynamics, 23 isolates were collected from kalanchoe fields in Gifu (from 2004-2009) and 16 were collected from strawberry and asparagus fields in Saga (from 2012-2013).

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 (*Cathtaranthus roseus)*	Aichi, Japan	1997	2
2	Chubu	GK10NI2SH	Periwinkle (*Cathtaranthus roseus)*	Gifu, Gifu, Japan	2010	1
3	Chubu	GK 08NI8S2	Periwinkle (*Cathtaranthus roseus)*	Gifu, Gifu, Japan	2008	1
4	Chubu	GK10NI1SH	Periwinkle (*Cathtaranthus roseus)*	Gifu, Gifu, Japan	2010	1
5	Chubu	OINB113SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	2
6	Chubu	OINB153	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	2
7	Chubu	OINB172	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	2
8	Chubu	OINB171SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	2
9	Chubu	OINB171	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	2
10	Chubu	OINB153SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	2
11	Chubu	OINB 113	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	2

12	Chubu	OINB 161	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2004	2
13	Chubu	OIOL0591R	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2005	2
14	Chubu	OINO 451 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2005	2
15	Chubu	OINO 451	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2005	2
16	Chubu	OIOLO 0581 R	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2005	2
17	Chubu	0705W21	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2007	2
18	Chubu	0805WJ21	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2008	1
19	Chubu	09E11294	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2009	1
20	Chubu	09E321 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2009	2
21	Chubu	09W422 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2009	2
22	Chubu	09WCRS1-1	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2009	2
23	Chubu	09W221 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	2009	2
24	Chubu	PHK6214 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	Unknown	2
25	Chubu	GK 4-11	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	Unknown	2
26	Chubu	PHKq-11	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	Unknown	2
27	Chubu	PGS1 SH	Kalanchoe (*Kalanchoe* sp.)	Katagata, Gifu, Japan	Unknown	2
28	Chubu	MAFF 712342	China doll (*Radermachera sinica)*	Ise, Mie	Unknown	2
29	Chubu	NBRC 30595	Strawberry (*Fragaria x ananassa*)	Shizuoka	1979	r
30	Chubu	MAFF 305926	Strawberry (*Fragaria x ananassa*)	Shizuoka, Japan	Unknown	2
31	Chubu	GF468	Strawberry (*Fragaria x ananassa*)	Gifu, Japan	2003	2
32	Chubu	GF524	Rose of Sharon *(Hibiscus syriacus)*	Ogaki, Gifu, Japan	2003	2
33	Kansai	CH00POIN 2	Poinsettia (*Euphorbia pulcherrima*)	Hyogo, Japan	2000	2
34	Kansai	CH00POIN3	Poinsettia (*Euphorbia pulcherrima*)	Hyogo, Japan	2000	2
35	Kansai	MAFF 239554	Poinsettia (*Euphorbia pulcherrima*)	Hyogo, Japan	2003	2
36	Kanto	CH08DAV11	*Euphorbia* sp.	Chiba, Japan	2008	2

37	Kanto	C23	Indian mallow (*Abutilon* sp.)	Chiba, Japan	2007	2
38	Kanto	C24	Indian mallow (*Abutilon* sp.)	Tateyama, Chiba, Japan	2007	2
39	Kanto	MAFF305795	African violet (*Saintpaulia goetzeana*)	Tachikawa, Tokyo, Japan	1987	2
40	Kanto	CH94AROE1	*Aloe vera*	Miyoshi, Chiba, Japan	1994	2[1]
41	Kanto	CH94AROE3	*Aloe vera*	Miyoshi, Chiba, Japan	1994	2[1]
42	Kanto	CH92ALS11	Peruvian lily (*Alstroemeria* sp.)	Kyonan, Chiba, Japan	1992	2[1]
43	Kanto	CH92ALS21	Peruvian lily (*Alstroemeria* sp.)	Kyonan, Chiba, Japan	1992	2[1]
44	Kanto	GUGC5631	Peruvian lily (*Alstroemeria* sp.)	Kyonan, Chiba, Japan	1992	2[1]
45	Kanto	CH93ANE1	Spanish marigold (*Anemone coronaria*)	Kimitsu, Chiba, Japan	1993	1[1]
46	Kanto	CH93ANE2	Spanish marigold (*Anemone coronaria*)	Kimitsu, Chiba, Japan	1993	1[1]
47	Kanto	CH 90-4	Zebra plant (*Aphelandra squarrosa)*	Chiba, Chiba, Japan	1990	2[1]
48	Kanto	CH90-9	Zebra plant (*Aphelandra squarrosa)*	Chiba, Japan	1990	2[1]
49	Kanto	CH90-6	Zebra plant (*Aphelandra squarrosa)*	Chiba, Japan	1990	2[1]
50	Kanto	CH89-44	*Bougenvillea* sp.	Kyonan, Chiba, Japan	1989	2[1]
51	Kanto	CH89-43	*Bougenvillia* sp.	Kyonan, Chiba, Japan	1989	2[1]
52	Kanto	C38	*Brodiaea* sp.	Chiba, Japan	2007	2
53	Kanto	MAFF 305796	Periwinkle (*Cathtaranthus roseus)*	Tokyo, Japan	1988	
54	Kanto	CH98Y1A	Yuzu (*Citrus junos*)	Futtsu, Chiba, Japan	1998	1[1]
55	Kanto	CH98U1A	Tangerine (*Citrus unshiu*)	Futtsu, Chiba, Japan	1998	1[1]
56	Kanto	MAFF 235436	*Daphne* sp.	Ibaraki, Tsukuba, Japan	1983	r
57	Kanto	CH95PHJ2	Winter daphne (*Daphne odora)*	Asahi, Chiba, Japan	1995	2[1]
58	Kanto	CH95PHJ1	Winter daphne (*Daphne odora)*	Asahi, Chiba, Japan	1995	2[1]
59	Kanto	CH87CWE1	*Dianthus* sp.	Wada, Chiba, Japan	1987	2[1]
60	Kanto	CH87-51	*Dianthus* sp.	Chikura, Chiba, Japan	1987	2[1]

61	Kanto	GUGC5562	*Dianthus* sp.	Chikura, Chiba, Japan	1987	2[1]
62	Kanto	CH87KTK1	Carnation (*Dianthus caryophyllus*)	Tomiura, Chiba, Japan	1987	2[1]
63	Kanto	CH87WG1	Carnation (*Dianthus caryophyllus*)	Wada, Chiba, Japan	1987	2[1]
64	Kanto	CH87CWG1	Carnation (*Dianthus caryophyllus*)	Wada, Chiba, Japan	1987	2[1]
65	Kanto	CH87-50	*Dianthus* sp.	Chiba, Japan	1987	2[1]
66	Kanto	C15	*Echium fastuosum*	Tateyama, Japan	2006	2
67	Kanto	C58	*Gerbera* sp.	Chiba, Japan	2008	2
68	Kanto	CH96HE1	English ivy (*Hedera helix*)	Kyonan, Chiba, Japan	1996	2[1]
69	Kanto	CH97HE11	English ivy (*Hedera helix*)	Maruyama, Chiba, Japan	1997	2[1]
70	Kanto	CH96HE2	English ivy (*Hedera helix*)	Kyonan, Chiba, Japan	1996	2[1]
71	Kanto	C26	Lavender (*Lavandula angustifolia*)	Chiba, Japan	2007	2
72	Kanto	CH99LK1	Lily (*Lilium hybrida)*	Kyonan, Chiba, Japan	1999	2[1]
73	Kanto	CH91KK4	Easter lily (*Lilium longiflorum*)	Kyonan, Chiba, Japan	1991	2[1]
74	Kanto	GUGC5567	Easter lily (*Lilium longiflorum*)	Kyonan, Chiba, Japan	1991	2[1]
75	Kanto	GUGC5630	*Limonium* sp.	Maruyama, Chiba, Japan	1991	2[1]
76	Kanto	GUGC5673	*Limonium* sp.	Maruyama, Chiba, Japan	1991	2[1]
77	Kanto	CH91-33	*Limonium* sp.	Maruyama, Chiba, Japan	1991	2[1]
78	Kanto	CH91-29	*Limonium* sp.	Maruyama, Chiba, Japan	1991	2[1]
79	Kanto	CH92ORN21	*Ornithogallum* sp.	Futtsu, Chiba, Japan	1992	2[1]
80	Kanto	CH92ORN11	*Ornithogallum* sp.	Futtsu, Chiba, Japan	1992	2[1]
81	Kanto	CH93ORN4	*Ornithogallum* sp.	Tateyama, Chiba, Japan	1993	2[1]
82	Kanto	GUGC5632	*Ornithogallum* sp.	Futtsu, Chiba, Japan	1992	2[1]
83	Kanto	MAFF 712287	*Viola tricolor*	Saitama, Japan	2006	1
84	Kanto	CH85PHP37	*Petroselinum crispum*	Maruyama, Chiba, Japan	1985	2[1]
85	Kanto	CH85PHP61	*Petroselinum crispum*	Maruyama, Chiba, Japan	1985	2[1]
86	Kanto	CH075STR81	Strawberry (*Fragaria x ananassa*)	Chiba, Japan	2007	2[1]

87	Kanto	CH91-1	*Strelitzia* sp.	Tateyama, Chiba, Japan	1991	2[1]
88	Kanto	CH91-4	*Strelitzia* sp.	Tateyama, Chiba, Japan	1991	2[1]
89	Kanto	CH91-3	*Strelitzia* sp.	Tateyama, Chiba, Japan	1991	2[1]
90	Kanto	CH91-2	*Strelitzia* sp.	Tateyama, Chiba, Japan	1991	2*
91	Kanto	GUGC5633	*Strelitzia* sp.	Chiba, Chiba, Japan	1991	2*
92	Kanto	MAFF 305939	*Nicotiana rustica*	Kanagawa, Japan	Unknown	r
93	Kanto	CH89-39	*Vanda* sp.	Tateyama, Chiba, Japan	1989	2[1]
94	Kanto	CH89-40	*Vanda* sp.	Tateyama, Chiba, Japan	1989	2[1]
95	Kanto	CH99TK2	Lili (*Lillium hybrida*)	Chiba	1999	Nr
96	Kyushu	SG12ASP1-1	Asparagus (*Asparagus officinalis*)	Saga, Japan	2012	2
97	Kyushu	SG12ASP1-2	Asparagus (*Asparagus officinalis*)	Saga, Japan	2012	2
98	Kyushu	SG12ASP2-1	Asparagus (*Asparagus officinalis*)	Saga, Japan	2012	2
99	Kyushu	SG12ASP1-3	Asparagus (*Asparagus officinalis*)	Saga, Japan	2012	r
100	Kyushu	SG12ASP2-2	Asparagus (*Asparagus officinalis*)	Saga, Japan	2012	2
101	Kyushu	SG13ASP1-2	Asparagus (*Asparagus officinalis*)	Saga, Japan	2013	2
102	Kyushu	SG13ASP1-1	Asparagus (*Asparagus officinalis*)	Saga, Japan	2013	2
103	Kyushu	SG13ASP1-3	Asparagus (*Asparagus officinalis*)	Saga, Japan	2013	1
104	Kyushu	MAFF 237653	Strawberry (*Fragaria x ananassa*)	Saga, Japan	1978	2
105	Kyushu	MAFF 242197	Strawberry (*Fragaria x ananassa*)	Saga, Japan	2004	2
106	Kyushu	SGPC 0503	Strawberry (*Fragaria x ananassa*)	Saga, Japan	Unknown	2
107	Kyushu	SGPY 2101	Strawberry (*Fragaria x ananassa*)	Saga, Japan	Unknown	2
108	Kyushu	SGPC 0502	Strawberry (*Fragaria x ananassa*)	Saga, Japan	Unknown	2

109	Kyushu	SGPC 04118	Strawberry (*Fragaria x ananassa*)	Saga, Japan	Unknown	2
110	Kyushu	SGPC 0501	Strawberry (*Fragaria x ananassa*)	Saga, Japan	Unknown	2
111	Kyushu	SGHP0002	Strawberry (*Fragaria x ananassa*)	Saga, Japan	Unknown	2
112	Kyushu	MAFF 305940	*Nicotiana rustica*	Kagoshima, Japan	1977	2
113	Kyushu	SE759	na	Saga, Japan		2
114	Kyushu	F03	na	Fukuoka, Japan	2006	1
115	Shikoku	MAFF 238154	Onion (*Allium cepa*)	Kochi, Japan	1999	1
116	Shikoku	NBRC 33191	Scallion (*Allium fistulosum*)	Kochi, Japan	1999	2[1]
117	Shikoku	NBRC 33190	Scallion (*Allium fistulosum*)	Kochi, Japan	1999	2[1]
118	Shikoku	MAFF 238152	*Lilium* sp.	Kochi, Japan	1999	2
119	Shikoku	NBRC 33193	*Lilium* sp	Kochi, Japan	1999	2[1]
120	Shikoku	NBRC 33192	Flame lily (*Gloriosa superba*)	Kochi, Japan	1999	2
121	Shouthern Island	MAFF 305797	*Dracaena* sp.	Hachijojima, Japan	1986	r
122	Shouthern Island	MAFF 305591	Papaya (*Carica papaya*)	Ogasawara, Japan	1986	2
123	Shouthern Island	MAFF 305799	Passion fruit (*Passiflora edulis*)	Hachijojima, Japan	1983	2
124	Shouthern Island	MAFF 305978	Passion fruit (*Passiflora edulis*)	Ogasawara, Japan	1988	2
125	Shouthern Island	MAFF 305590	Tomato (*Solanum lycopersicum)*	Ogasawara, Japan	1986	r
126	Taiwan	NBRC 31425	Onion (*Allium cepa*)	Taiwan	1984	1[1]
127	Taiwan	NBRC 31423	Pineapple *(Annanas comosus*)	Taiwan	1984	1[1]
128	Taiwan	NBRC 31419	Papaya (*Carica papaya*)	Taiwan	1984	2[1]
129	Taiwan	NBRC 31416	Tomato (*Solanum lycopersicum*)	Taiwan	1984	2[1]
130	Indonesia	TBC GTS	Tobacco (*Nicotiana rustica*)	Central Java, Indonesia	2016	1
131	Indonesia	AA 129D 2	Pineapple *(Annanas comosus*)	Lampung, Indonesia	2016	1
132	Indonesia	AA 71A S1	Pineapple *(Annanas comosus*)	Lampung, Indonesia	2016	2
133	Indonesia	AA 114K HS 2	Pineapple *(Annanas comosus*)	Lampung, Indonesia	2016	1
134	Indonesia	AA 71A 2	Pineapple *(Annanas comosus*)	Lampung, Indonesia	2016	2

135	Indonesia	AA 36G	Pineapple *(Annanas comosus)*	Lampung, Indonesia	2016	1
136	Indonesia	AA 71A 3	Pineapple *(Annanas comosus)*	Lampung, Indonesia	2016	1
137	USA	CBS 535.92	Soil	USA		1[1]
138	USA	CBS 534.92	Soil	USA		2[1]

[1]) Mating type data was provided on the origin of the isolates
Nr: no mating reaction; Unknown: information not available

Phytophthora nicotianae was isolated by plating infected plant tissues onto selective NARM medium as previously described (Morita and Tojo 2007). The resultant mycelia were then identified at a molecular level by sequencing of the internal transcribed spacer (ITS) region and cytochrome c oxidase 1 (*COX I*) gene (Robideau et al. 2011). The isolates were categorized into nine population groups based on their geographical origin: five populations from Japan main island (Chubu, Kansai, Kanto, Kyushu, Shikoku) and the Southern Islands, 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 the mating zone was formed and the development of antheridia and oogonia was observed.

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 trinucleotide and tetranucleotide. 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 ºC (optimum at 58 ºC), 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 analysed 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 web-base program insilico.ehu.eus (San Millan et al. 2013). Amplified fragments were cloned using the TOPO TA cloning kit (Invitrogen) and then sequenced in order 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). The purified PCR product was sequenced using the M13M4 primer for amplification by the BigDye Sequence Terminator Kit on an ABI3500 automated sequencer.

Microsatellite genotyping

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

The total genome DNA was extracted using PrepMan Ultra Reagent (Applied Biosystem) and amplified using all selected primers under the following conditions: 1

cycle of 94 ºC for 5 min, followed by 35 cycles of 94 ºC for 30 s, then 58 ºC for 30 s, 72 ºC for 30 s and a final extension at 72 ºC 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 x PCR Buffer (plus magnesium, Takara Bio Inc.), 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), 0.1 µl rTaq polymerase (Takara Bio Inc.), and 10.9 µl ddH_2O. PCR amplification products were assessed by GelRed staining (Biotium) using 2% Agarose gels in 0.5× Tris-Acetate-EDTA buffer and visualized under UV light.

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

Population structure analysis

The population structure was analysed by cluster analysis, which was performed by estimating the probability of genotypes being distributed into K number of cluster using STRUCTURE v. 2.3.4. (Pritchard, Stephens, and Donnelly 2000; Falush, Stephens, and Pritchard 2003, 2007; Hubisz et al. 2009) with an admixture model without prior population information and 200000 MCMC. Eight independent runs were performed for each K= 1-20. The optimal number of K was decided by STRUCTURE HARVESTER (Earl and vonHoldt 2012) and matched from the 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 2012) was used for phylogenetic analysis using a neighbor-joining algorithm on MEGA 6.0 (Tamura et al. 2006). A statistical analysis of the mating pattern within the population

was conducted by Analysis of Molecular Variance (AMOVA) using GenAlex 5.6.3 (Peakall and Smouse 2006, 2012).

Results

Development of microsatellite marker

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 exhibited multiple alleles were selected for this 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.

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

Locus	Repeat Motif	Primer sequence	Annealing temperature (°C)	Flourescent Label	Alleles	
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

In total, 39 alleles were detected from six loci, ranging from four (TAA) to 11 (GTA), with an average number of alleles per locus of 6.5 (Table 3). The highest number of alleles was 11, in locus GTA. This locus was also the most informative, as it had the highest Shanon's information index (I= 1.838). Two out of six alleles had significantly higher observed heterozygosity while the rest were significantly lower. All of the loci significantly differed from HWE.

Table 3 Microsatellite characteristics

Locus	Na	Ne	I	Ho	He	P	Fst
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: Shanon's information index; Ho: observed heterozygosity; He: expected heterozygosity; P: P-value on Hardy-Weinberg's equilibrium; Fst: fixation index

Mating type distribution

From 138 isolates, 21 isolates were identified as the A1 mating type, 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). In the kalanchoe and asparagus farms, the A1 and A2 mating types were isolated on a different year, while both mating types were isolated on the same year in Indonesian pineapple fields (Table 1).

Phylogenetic analysis

The phylogenetic tree constructed with the neighbor joining algorithm revealed five major clades (Fig. 5). The isolates collected from the Kanto area were scattered on all clades in the phylogenetic tree as well as the isolates collected from Taiwan, Shikoku, and Southern Island. The isolates collected from the same geographic origin but from different host plants, were found to have distant relationships and this was also the case for the isolates collected from the same host plants but with different geographic origin. However, several isolates were observed to have the same genotype to other isolates collected from the same host and geographic origin. These were three isolates (working number 76-78) collected 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) collected from bird of paradise flower.

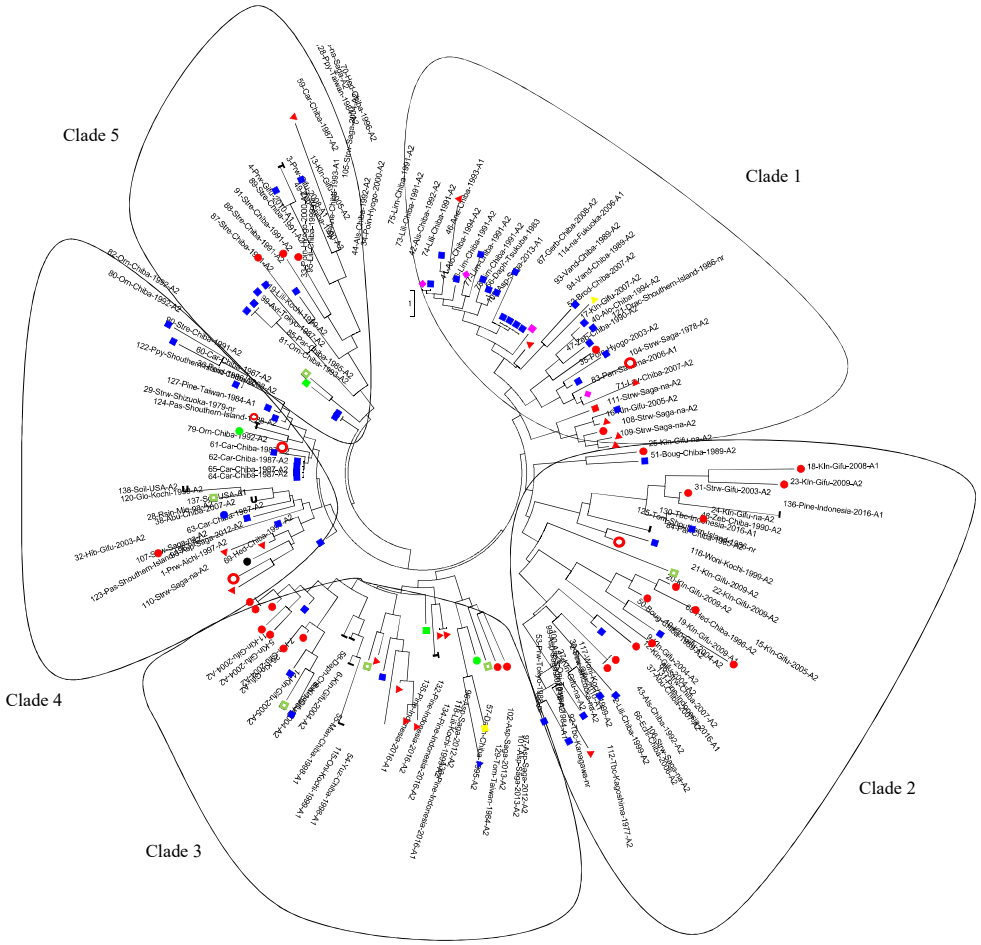

Figure 5. Phylogenetic analysis of *P. nicotianae* populations. Each symbol represent geographic origin

○ Chubu: ● Aichi; ● Gifu; ● Mie; ● Shizuoka; ◆ Kansai (Hyogo); ■ Kanto: ■ Chiba; ■ Tokyo; ■ Tsukuba; ■ Saitama; ■ Kanagawa; ▲Kyushu: ▲ Saga; ▲ Kagoshima; ▲ Fukuoka; ○ Southern Island; □ Shikoku (Kochi); **I**: Indonesia; **U**: USA; **T**: Taiwan. Abbreviations for host: 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: Tobcco; Abu: ; Echi: ; Prw: Periwinkle; Woni: Whelsh onion; Oni: Onion; Daph: Daphne; Pas: Passion fruit; Car: Carnation; Hib: Hibiscus; Rsin: ; Orn: Ornithogallum; Zeb: zebra plant; Hed: Hedera (english ivy).

The isolates collected from the same host and geographic origin in different years were observed to have different genotypes. The isolates collected from kalanchoe in Gifu in 2004 were grouped in clades 2 and 3 (No 10, 9, 12; and 5-8, 11, respectively), whilst the isolates collected in 2007 (No. 17) were found in the clade 1. Isolates collected from *Ornitogallum* sp. in Chiba in 1992 (No. 80 and 82) were grouped in clade 4 while the isolates collected in 1993 (No. 81) were grouped in clade 5. Interestingly, the isolate (No. 81) from *Ornithogallum* sp. had the same genotype to that of the 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 (Fig. 5). phylogenic analysis, these clusters did not reflect the geographical distribution of the populations (Fig. 5,6). The first and second clusters (orange and blue color) were found in all populations from Japan (Main Island) and were predominant in populations from Kansai and Shikoku but absent in those from Indonesia and the Southern Islands. The second cluster (blue) was prevalent in populations from Shikoku, Taiwan, and Indonesia. The third cluster (red) was predominant in populations from Shikoku, Taiwan, and USA but only moderately in those from Japan. The fourth group (green) was identified in populations from Japan, Taiwan, and Indonesia but rarely in those from the USA. The fifth cluster (yellow) was predominant in the Southern Island and USA populations.

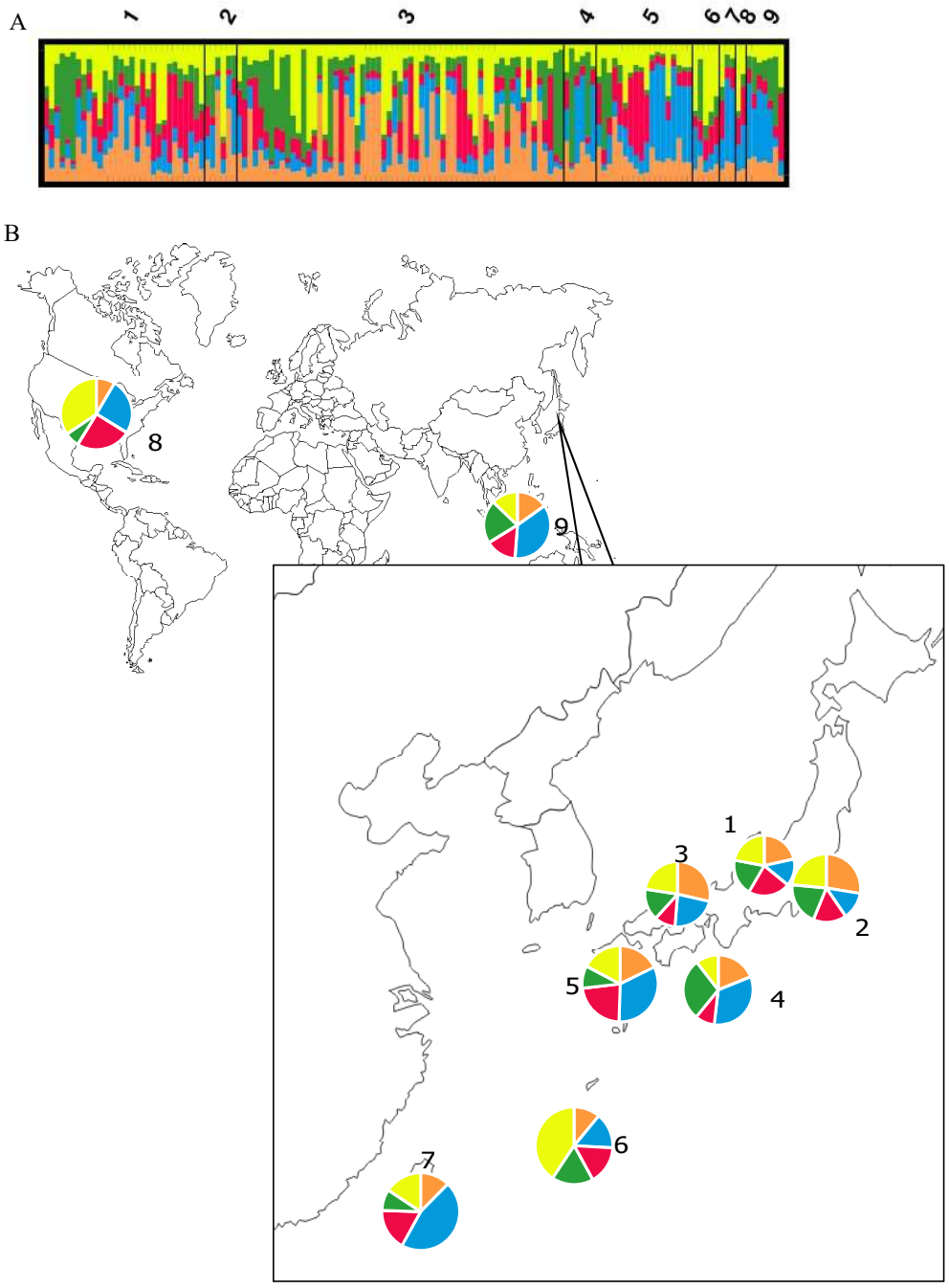

Figure 6. Cluster analysis of *Phytophthora nicotianae* using STRUCTURE v 2.3.4 (A). Genotypic clustering in each population (1: Chubu; 2: Kanto; 3: Kansai; 4: Shikoku; 5:Kyushu; 6: Southern Island; 7: Taiwan 8: USA; 9: Indonesia;). (B). The proportion of genotypic clusters in each population

The analysis of molecular variance (AMOVA) of microsatellite genotype data showed that the isolates have low diversity among the population (3%) but high diversity among individuals within the population (Table 4). The low fixation index (F_{ST}) number 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.

Table 4. Summary of analysis of molecular variance (AMOVA)

Source	df	SS	MS	Est. Var.	%	Fstatistic	P-value
Among Pops	8	28.014	3.502	0.064	3%	Fst = 0.033	0.087
Within Pops	267	500.812	1.876	1.876	97%		
Total	275	528.826		1.940	100%		

df: degree of freedom; SS: sum of square; MS: mean of square; Est. Var.: estimated variance; %: percentage of variance; Fst: fixation index.

Discussion

Previous studies on the population genetics of *P. nicotianae* have been based on mitochondrial and nuclear DNA. Importantly, these studies have used isolates from Australia and the USA, and so little was known about *P. nicotianae* populations from Japan. An analysis of the population genetics of *P. nicotianae* in Japan could provide a better understanding of how the pathogen is likely to emerge at a more local level.

In the present study, novel microsatellite markers were developed to analyze the population genetics of *P. nicotianae* in Japan. The microsatellite markers were harvested from full genome sequence data

(https://olive.broadinstitute.org/projects/phytophthora_parasitica) using Tandem Repeat Finder (Benson 1999). The availability of genome sequence data plays an important role in developing microsatellite primers. If the genome sequence is unavailable, suppression and tail PCR is required to develop microsatellite markers, which is very costly and time consuming (Yin-Ling et al. 2009). The markers were then used to amplify six loci from 138 isolates collected 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). In total, 39 alleles were amplified from six microsatellite loci. This number of alleles is much higher than has previously been reported for *P. infestans* (Montarry et al. 2010) and *P. sojae* (M. Wu et al. 2017). This is due to the broader host range of *P. nicotianae*, as compared to *P. infestans* and *P. sojae.*

The pathogen isolated from kalanchoe in Gifu was scattered across several clades of the phylogenetic tree (Fig. 4). These isolates were collected from the same farm. In this case, the different year of isolation was a significant factor. The isolates from 2004, 2008, and 2009 were grouped into clades 2 and 3 on the phylogenetic tree, whilst the isolates from 2005 and 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 of A2 type, thus preventing sexual recombination.

By contrast, the grouping of isolates collected from the Saga prefecture tended to be according to the mating type and host, rather than by their year of isolation. The A2 mating type isolates collected from asparagus were grouped in clade 3, even though they were collected during a different year, whilst the A1 mating type was in the clade 1. The isolates collected from pineapple in Indonesia were also grouped into a single clade

(Clade 3) and differed from the isolates collected from pineapple in Taiwan (Clade 4). These results show that the sources of infection would be 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 and so raising the likelihood of the creation of novel genotypes with new combinations of traits through natural selection, and of masking deleterious mutations caused by inbreeding (Verhoeven et al. 2011). This is likely to be due to the choice of host plants used in this study, the majority of which were ornamental. It has been previously reported that isolates from ornamental species were 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 of genotypic clusters and geographical origins are common in demographic analysis 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. The high number of genotypic clusters of a population is linear to the percentage of variance among the individuals of the population. This was confirmed by AMOVA tests which showed 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 ($\Delta K = 9$). The low number

of F_{ST} value (0.033) and associated p-value of 0.08, revealed that there was no significant genetic differentiation among groups. The undifferentiated population indicates the possibility of sharing genetic materials between the populations (Ma, Ji, and Zhang 2015). This explains why there was no strong geographical structuring in the Japanese *P. nicotianae* populations.

The lack of strong geographical structure of the *P. nicotianae* population 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 would be possible for the pathogen to be inadvertently transported with agricultural products or via water courses. Both the phylogenetic analysis and population structure results agree with the 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. Whilst 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 in order to understand the route(s) of migration by *P. nicotianae.*

CHAPTER II

Population structures of the water-borne plant pathogen *Phytopythium helicoides* reveal its possible origins and transmission modes in Japan

Developments in agriculture and global trading, and innovations in plant breeding can lead to the wide distribution of genetically homogeneous crops. These factors, along with the tendency of farmers to cultivate the same varieties over large areas could accelerate the pace of pathogen dispersal to new areas. Both human mediated introduction and natural dispersal mechanisms can lead to the spread of pathogens (Barrès et al. 2012). Humans can accidentally introduce pathogens to new areas through the transport of infected agricultural materials. In Japan, the widespread use of hydroponic culture systems could lead to the rapid spread of water borne diseases. *Phytopythium helicoides* is a soil and water-borne pathogen that causes serious problems in a variety of horticultural crops. This pathogen produces large numbers of zoospores that serve as a secondary inoculum source. Thus, *P. helicoides* can spread rapidly in hydroponic farming systems.

Phytopythium is a recently established genus consisting of species that were formerly classified in the *Pythium* phylogenetic clade K (Bala et al. 2010). These species are morphologically similar to *Pythium* spp. but are genetically closer to *Phytophthora* spp. (de Cock et al. 2015). *Phytopythium helicoides* is representative species that produces papillate sporangia like *Phytophthora spp*. It also has a *Pythium*-like zoospore

discharge mechanism whereby the plasma flows out of the sporangium through a discharge tube to form a plasma-filled vesicle at the tip. *Phytopythium helicoides* is a high temperature tolerant pathogen that was first isolated from dahlia roots in 1930 by Drechsler in the USA (Kageyama et al. 2002). After that, there were few reports of the pathogen until it was isolated on miniature rose in Gifu, Japan (Kageyama et al. 2002). Since then it has caused root and stem rot in other agricultural important plants in Japan, including kalanchoe, kiwi, and strawberry (Watanabe et al. 2007; Ishiguro et al. 2014). Furthermore, it has recently caused root rot and stem rot in bell pepper and pistachio in the USA (Chellemi et al. 2000), citrus mandarin and kiwi fruit in china (X. R. Chen et al. 2016; Wang et al. 2015), and rose in Korea (Han et al. 2010). These diseases suddenly appeared in areas where they had not occurred before, however, the sources of the inoculum have not been identified.

Previous studies on the genetic diversity of *P. helicoides* revealed high variability in the rDNA ITS region within a single isolate, suggesting that this pathogen will undergo cross-breeding even though it is a homothallic species (Kageyama et al. 2007). Despite its ability to cross-breed, this pathogen can also lose its ability to produce sexual structures. Asexual strains of *P. helicoides* produce no oospores but abundant sporangia. The sporangium is an effective survival structure in the greenhouse, where high humidity and temperatures are maintained throughout the year (Kageyama et al. 2003). The high variability among isolates of *P. helicoides* could indicate that new strains will emergence in the future.

Understanding the genetic variability in a pathogen could help in the development of effective disease management strategies. Studies that address the microevolution and

population structure of a pathogen are necessary to predict its adaptation and migration abilities. A pathogen that has high genetic diversity and high mobility is likely to adapt to environmental change. There are many methods for studying population genetics at the molecular level, and they are generally based on DNA polymorphisms such as single nucleotide polymorphisms or microsatellites. Microsatellites (also called simple sequence repeats) are very convenient for molecular studies involving PCR because they are codominant, multiallelic, and highly polymorphic, and only small amounts of DNA are needed for PCR analysis (Yin-Ling et al. 2009). We used microsatellites as a primary source of genotyping data in this study. Our aims were to (i) develop reliable microsatellite markers for *P. heliocoides*; (ii) identify the main genotypic clusters of *P. helicoides* in Japan; (iii) calculate the genetic diversity within the *P. helicoides* populations, and (iv) understand the transmission modes of *P. helicoides* across Japan.

Materials and methods

Isolates and DNA extraction

In total, 232 isolates of *P. helicoides* were selected from the Gifu University Culture Collection and used in this study (Table 5). Of these, 229 were collected from 19 geographical regions in 19 prefectures of Japan (Fig. 6), two were from the USA, and one came from the Netherlands. Most of the isolates were collected from the plants, soil, or water used in the production of horticultural and ornamental crops. However, 20 isolates were collected from natural environments outside agricultural areas. To understand the population structures within local areas, we studied 49 isolates from several poinsettia farms in Aichi, 30 isolates from six miniature rose farms in Gifu, and 22 isolates from

three cutting rose farms in Shizuoka. All isolates were stored on corn meal agar medium at room temperature until DNA extraction.

Table 5. Isolates of *Pythopythium helicoides* used in this study

Number	Isolates	Origin	Farm	Host	Sexuality	Year
1	GUCC 5208	Aichi	KZ	Poinsettia	Sexual	2011
2	GUCC 5209	Aichi	KZ	Poinsettia	Sexual	2011
3	GUCC 5210	Aichi	KZ	Poinsettia	Sexual	2011
4	GUCC 5211	Aichi	TY	Poinsettia	Sexual	2011
5	GUCC 5212	Aichi	HR	Poinsettia	Sexual	2012
6	GUCC 5213	Aichi	TY	Poinsettia	Sexual	2012
7	GUCC 5214	Aichi	TY	Poinsettia	Sexual	2012
8	GUCC 5215	Aichi	TY	Poinsettia	Sexual	2012
9	GUCC 5216	Aichi	TY	Poinsettia	Sexual	2012
10	GUCC 5217	Aichi	TY	Poinsettia	Sexual	2012
11	GUCC 5218	Aichi	TY	Poinsettia	Sexual	2012
12	GUCC 5219	Aichi	TY	Poinsettia	Sexual	2012
13	GUCC 5220	Aichi	TY	Poinsettia	Sexual	2012
14	GUCC 5221	Aichi	TY	Poinsettia	Sexual	2012
15	GUCC 5222	Aichi	TY	Poinsettia	Sexual	2012
16	GUCC 5223	Aichi	TY	Poinsettia	Sexual	2012
17	GUCC 5224	Aichi	TY	Poinsettia	Sexual	2012
18	GUCC 5225	Aichi	TY	Poinsettia	Sexual	2012
19	GUCC 5226	Aichi	TY	Poinsettia	Sexual	2012
20	GUCC 5227	Aichi	TY	Poinsettia	Sexual	2012
21	GUCC 5228	Aichi	TY	Poinsettia	Sexual	2012
22	GUCC 5229	Aichi	TY	Poinsettia	Sexual	2012
23	GUCC 5230	Aichi	TY	Poinsettia	Sexual	2012

24	GUCC 5231	Aichi	TY	Poinsettia	Sexual	2012
25	GUCC 5232	Aichi	TY	Poinsettia	Sexual	2012
26	GUCC 5233	Aichi	TY	Poinsettia	Sexual	2012
27	GUCC 5234	Aichi	TY	Poinsettia	Sexual	2012
28	GUCC 5235	Aichi	TY	Poinsettia	Sexual	2012
29	GUCC 5236	Aichi	TY	Poinsettia	Sexual	2012
30	GUCC 5237	Aichi	TY	Poinsettia	Sexual	2012
31	GUCC 5238	Aichi	TY	Poinsettia	Sexual	2012
32	GUCC 5239	Aichi	TY	Poinsettia	Sexual	2012
33	GUCC 5240	Aichi	TY	Poinsettia	Sexual	2012
34	GUCC 5241	Aichi	TY	Poinsettia	Sexual	2012
35	GUCC 5242	Aichi	TY	Poinsettia	Sexual	2012
36	GUCC 5243	Aichi	TY	Poinsettia	Sexual	2012
37	GUCC 5244	Aichi	TY	Poinsettia	Sexual	2012
38	GUCC 5245	Aichi	TY	Poinsettia	Sexual	2012
39	GUCC 5246	Aichi	TY	Poinsettia	Sexual	2012
40	GUCC 5247	Aichi	TY	Poinsettia	Sexual	2012
41	GUCC 5248	Aichi	TY	Poinsettia	Sexual	2012
42	GUCC 5249	Aichi	TY	Poinsettia	Sexual	2012
43	GUCC 5250	Aichi	TY	Poinsettia	Sexual	2013
44	GUCC 5251	Aichi	TY	Poinsettia	Sexual	2013
45	GUCC 5252	Aichi	TY	Poinsettia	Sexual	2013
46	GUCC 5253	Aichi	TY	Poinsettia	Sexual	2013
47	GUCC 5254	Aichi	TY	Poinsettia	Sexual	2013
48	GUCC 5255	Aichi	TY	Poinsettia	Sexual	2013
49	GUCC 5256	Aichi	TY	Poinsettia	Sexual	2013
50	Ai12022H1	Aichi	HR	Soil of miniature rose	Sexual	2013

51	Ai130223H1	Aichi	HR	Soil of miniature rose	Sexual	2013
52	Ai130213H2	Aichi	HR	Soil of miniature rose	Sexual	2013
53	Ai130417H2	Aichi	HR	Miniature rose	Sexual	2013
54	GUCC 5066a	Aichi	AK	Cutting Rose	Asexual	2000
55	GUCC 5067a	Aichi	AK	Cutting Rose	Asexual	2000
56	GUCC 5068a	Aichi	AK	Cutting Rose	Asexual	2000
57	GUCC 5069a	Aichi	AK	Cutting Rose	Asexual	2000
58	GUCC 5148	Aichi	TH	Cutting Rose	Sexual	2008
59	GUCC 5167	Aichi	TH	Cutting Rose	Sexual	2008
60	GUCC 5168	Aichi	TH	Cutting Rose	Sexual	2008
61	GUCC 5169	Aichi	TH	Cutting Rose	Sexual	2008
62	GUCC 5151	Aichi	TH	Water	Sexual	2008
63	GUCC 5170	Aichi	TH	Water	Sexual	2008
64	1410CU15	Aichi	-	Water sludge	Sexual	2014
65	K9b18-98	Aichi	-	Irrigation water	Sexual	2008
66	K9b18-116	Aichi	-	Irrigation water	Sexual	2009
67	T5a2-127	Aichi	-	Irrigation water	Sexual	2008
68	K9b16-102	Aichi	-	Irrigation water	Sexual	2008
69	K9b16-132	Aichi	-	Irrigation water	Sexual	2009
70	k8b16-120	Aichi	-	Drain water	Sexual	2008
71	K9b18-105	Aichi	-	Irrigation water	Sexual	2008
72	GUCC 5135	Fukui	RY	Soybean	Asexual	2004
73	GUCC 5171	Fukui	RY	Soybean	Asexual	2004
74	GUCC 5001	Gifu	GD	Rose	Asexual	1996
75	GUCC 5002	Gifu	GD	Rose	Asexual	1996
76	GUCC 5003	Gifu	GD	Rose	Asexual	1996
77	GUCC 5004	Gifu	GD	Rose	Asexual	1996

78	GUCC 5005	Gifu	GD	Rose	Asexual	1996
79	GUCC 5006	Gifu	GD	Rose	Asexual	1996
80	GUCC 5007	Gifu	KZ	Miniature rose	Sexual	1996
81	GUCC 5008	Gifu	KZ	Miniature rose	Sexual	1996
82	GUCC 5009	Gifu	KZ	Miniature rose	Sexual	1996
83	GUCC 5010	Gifu	KZ	Miniature rose	Sexual	2000
84	GUCC 5011	Gifu	KZ	Miniature rose	Sexual	2000
85	GUCC 5012	Gifu	KZ	Miniature rose	Sexual	2000
86	GUCC 5013	Gifu	SN	Miniature rose	Sexual	1996
87	GUCC 5014	Gifu	SN	Miniature rose	Sexual	1996
88	GUCC 5015	Gifu	SN	Miniature rose	Sexual	1996
89	GUCC 5016	Gifu	SN	Miniature rose	Sexual	2000
90	GUCC 5017	Gifu	SN	Miniature rose	Sexual	2000
91	GUCC 5018	Gifu	SN	Miniature rose	Sexual	2000
92	GUCC 5020	Gifu	SN	Miniature rose	Sexual	2000
93	GUCC 5021	Gifu	SN	Miniature rose	Sexual	2000
94	GUCC 5022	Gifu	IN	Miniature rose	Sexual	1999
95	GUCC 5023	Gifu	IN	Miniature rose	Sexual	1999
96	GUCC 5024	Gifu	IN	Miniature rose	Sexual	1999
97	GUCC 5025	Gifu	IN	Miniature rose	Asexual	2000
98	GUCC 5026	Gifu	IN	Miniature rose	Asexual	2000
99	GUCC 5027	Gifu	IN	Miniature rose	Asexual	2000
100	GUCC 5028	Gifu	IN	Miniature rose	Asexual	2000
101	GUCC 5029	Gifu	IN	Miniature rose	Sexual	2000
102	GUCC 5030	Gifu	IN	Miniature rose	Sexual	2000
103	GUCC 5031	Gifu	IN	Miniature rose	Sexual	2000
104	GUCC 5032	Gifu	MZ	Kalanchoe	Sexual	2000
105	GUCC 5033	Gifu	MZ	Kalanchoe	Sexual	2000
106	GUCC 5034	Gifu	MZ	Kalanchoe	Sexual	2000

107	GUCC 5035	Gifu	KT	Kalanchoe	Sexual	2001
108	GUCC 5036	Gifu	KT	Kalanchoe	Sexual	2001
109	GUCC 5037	Gifu	KT	Kalanchoe	Sexual	2001
110	GUCC 5038	Gifu	MZ	Miniature rose	Sexual	2001
111	GUCC 5039	Gifu	ON	Miniature rose	Sexual	2002
112	GUCC 5040	Gifu	ON	Miniature rose	Sexual	2002
113	GUCC 5041	Gifu	ON	Miniature rose	Sexual	2002
114	GUCC 5042	Gifu	GF	Strawberry	Sexual	2002
115	GUCC 5044	Gifu	TJ	Strawberry	Sexual	2004
116	GK12Ro1SH	Gifu	TK	Miniature rose	Sexual	2012
117	GK12Ro2SH	Gifu	TK	Miniature rose	Sexual	2012
118	GK12RosSH	Gifu	TK	Miniature rose	Sexual	2012
119	GK12Ro7SH	Gifu	SN	Miniature rose	Sexual	2012
120	GK12Ro8SH	Gifu	IN	Miniature rose	Sexual	2012
121	GK12Ro9SH	Gifu	SN	Miniature rose	Sexual	2012
122	GK12Ro10SH	Gifu	IN	Miniature rose	Sexual	2012
123	GK12Ro12-1FSH	Gifu	KZ	Miniature rose	Sexual	2012
124	GK12Ro3SH	Gifu	TK	Miniature rose	Sexual	2012
125	GK12Ros2-2	Gifu	TK	Miniature rose	Sexual	2012
126	GK12Ros2-21	Gifu	TK	Miniature rose	Sexual	2012
127	GK12Ros1-22	Gifu	TK	Miniature rose	Sexual	2012
128	GK14Ro2SH	Gifu	IN	Miniature rose	Sexual	2014
129	GK16Sed15	Gifu	-	Sedum	Sexual	2016
130	GKSed1SH	Gifu	-	Sedum	Sexual	2015
131	GUCC 5147	Gifu	-	Poinsettia	Sexual	2004
132	GUCC 5179	Gifu	-	Nagara water	Sexual	2001
133	GUCC 5192	Gifu	-	Soil of strawberry	Sexual	2011
134	OiLo581R	Gifu	KT	Kalanchoe	Sexual	2005

135	OiLo591R	Gifu	KT	Kalanchoe	Sexual	2005
136	OiLoJ8-IR	Gifu	KT	Kalanchoe	Sexual	2005
137	GUCC 5137	Iriomote, Okinawa		Water	Sexual	2007
138	GUCC 5138	Iriomote, Okinawa		Water	Sexual	2007
139	GUCC 5139	Iriomote, Okinawa		Water	Sexual	2007
140	GUCC 5140	Iriomote, Okinawa		Water	Sexual	2007
141	GUCC 5141	Iriomote, Okinawa		Water	Sexual	2007
142	GUCC 5142	Iriomote, Okinawa		Water	Sexual	2007
143	GUCC 5155	Iriomote, Okinawa		Water	Sexual	2007
144	GUCC 5156	Iriomote, Okinawa		Water	Sexual	2007
145	GUCC 5076	Kagawa		Chrysanthemum	Sexual	2005
146	GUCC 5077	Kagawa		Chrysanthemum	Sexual	2005
147	GUCC 5165	Kagawa		Chrysanthemum	Sexual	2005
148	KP2013-10.1	Kagawa		Strawberry	Sexual	2013
149	GUCC 5160	Rebun Island		Water	Sexual	2008
150	GUCC 5108	Mie		Soil, fig field	Sexual	1982
151	GUCC 5075	Mie		Rhododendron	Sexual	2000
152	MGR01	Miyagi		Cutting Rose	Sexual	2012
153	MGR03	Miyagi		Cutting Rose	Sexual	2012
154	GUCC 5143	Nagano		Strawberry	Sexual	2004
155	GUCC 5106	Nara		Soil, spinach field	Sexual	1982
156	GUCC 5107	Nara		Soil, pinto bean field	Sexual	1982

157	GUCC 5109	Nara		Soil, strawberry field	Sexual	1982
158	GUCC 5194	Nara		Strawberry soil	Sexual	2011
159	GUCC 5104	Netherlands		Cutting rose	Sexual	1998
160	GUCC 5072	Niigata		Cutting Rose	Sexual	2000
161	GUCC 5045	Oita	KK	Cutting Rose	Sexual	2000
162	GUCC 5046	Oita	KJ	Cutting Rose	Sexual	2000
163	GUCC 5047	Oita	KJ	Cutting Rose	Sexual	2000
164	GUCC 5048	Oita	KJ	Cutting Rose	Sexual	2000
165	GUCC 5049	Oita	KJ	Cutting Rose	Sexual	2000
166	GUCC 5050	Oita	KJ	Cutting Rose	Sexual	2000
167	GUCC 5051	Oita	KJ	Cutting Rose	Sexual	2000
168	GUCC 5052	Oita	KJ	Cutting Rose	Sexual	2000
169	GUCC 5053	Oita	KJ	Cutting Rose	Sexual	2000
170	GUCC 5054	Oita	KJ	Cutting Rose	Sexual	2000
171	GUCC 5056	Oita	KJ	Cutting Rose	Sexual	2000
172	GUCC 5057	Oita	KJ	Cutting Rose	Sexual	2000
173	GUCC 5058	Oita	KJ	Cutting Rose	Sexual	2000
174	GUCC 5059	Oita	BP	Cutting Rose	Sexual	2000
175	GUCC 5060	Oita	BP	Cutting Rose	Sexual	2000
176	GUCC 5061	Oita	BP	Cutting Rose	Sexual	2000
177	GUCC 5074	Oita	BP	Cutting Rose	Sexual	2000
178	GUCC 5195	Saga		Strawberry soil	Sexual	2011
179	GUCC 5196	Saga		Strawberry soil	Sexual	2011
180	GUCC 5112	Shizuoka		Rose	Sexual	2005
181	GUCC 5123	Shizuoka		Gerbera	Sexual	2008
182	GUCC 5114	Shizuoka	FK	Cutting Rose	Sexual	2005
183	GUCC 5119	Shizuoka	KG	Strawberry	Sexual	2004
184	GUCC 5120	Shizuoka	KG	Strawberry	Sexual	2004

185	GUCC 5122	Shizuoka	KG	Strawberry	Sexual	2006
186	GUCC 5111	Shizuoka	SM	Cutting Rose	Sexual	2006
187	GUCC 5118	Shizuoka	SM	Cutting Rose	Sexual	2004
188	GUCC 5073	Shizuoka	KW	Cutting Rose	Asexual	2000
189	GUCC 5086	Shizuoka	KW	Cutting Rose	Sexual	2004
190	GUCC 5087	Shizuoka	KW	Cutting Rose	Sexual	2004
191	GUCC 5089	Shizuoka	KW	Cutting Rose	Sexual	2004
192	GUCC 5090	Shizuoka	FJ	Cutting Rose	Sexual	2004
193	GUCC 5105	Shizuoka		Strawberry	Sexual	1973
194	GUCC 5113	Shizuoka		Cutting Rose	Sexual	na
195	SR12-41	Shizuoka		Water of cutting rose field	Sexual	2012
196	SR-12-43	Shizuoka		Cutting Rose	Sexual	2012
197	SR12-44	Shizuoka		Cutting Rose	Sexual	2012
198	SR12-47	Shizuoka		Cutting Rose	Sexual	2012
199	SR12-48	Shizuoka		Cutting Rose	Sexual	2012
200	SR12-49	Shizuoka		Cutting Rose	Sexual	2012
201	SR12-52a	Shizuoka		Cutting Rose	Asexual	2012
202	SR12-60	Shizuoka		Cutting Rose	Sexual	2012
203	SR12-61a	Shizuoka		Cutting Rose	Asexual	2012
204	SR12-64a	Shizuoka		Cutting Rose	Asexual	2012
205	SR12-66	Shizuoka		Cutting Rose	Sexual	2012
206	SR16-63CY	Shizuoka		Cutting Rose	Sexual	2016
207	GUCC 5115	Shizuoka		Cutting Rose	Sexual	2003
208	GUCC 5116	Shizuoka		Cutting Rose	Sexual	2004
209	GUCC 5197	Shizuoka		Soil of strawberry	Sexual	2011
210	GUCC 5198	Shizuoka		Soil of strawberry	Sexual	2011

211	GUCC 5199	Shizuoka		Soil of strawberry	Sexual	2011
212	SPKSO4A1	Shizuoka		Soil of strawberry	Sexual	2005
213	SPR07-1	Shizuoka		Rose	Sexual	2007
214	GUCC 5133	Tochigi	MS	Strawberry	Sexual	2007
215	GUCC 5128	Tochigi	ST	Strawberry	Sexual	2007
216	GUCC 5129	Tochigi	ST	Strawberry	Sexual	2007
217	GUCC 5079	Toyama		Cutting Rose	Sexual	Unknown
218	GUCC 5080	Toyama		Cutting Rose	Sexual	Unknown
219	GUCC 5081	Toyama		Cutting Rose	Sexual	Unknown
220	GUCC 5096	USA		Common bean	Sexual	1931
221	GUCC 5095	USA		Honda soil	Sexual	1962
222	GUCC 5070	Wakayama		Cutting Rose	Sexual	2004
223	GUCC 5071	Wakayama		Cutting Rose	Sexual	2004
224	YGS378SH	Yamagata		Soil of strawberry	Sexual	Unknown
225	GUCC5201	Yamanashi	YN	Soil of strawberry	Sexual	2011
226	GUCC5202	Yamanashi	YN	Soil of strawberry	Sexual	2011
227	GUCC5203	Yamanashi	YN	Soil of strawberry	Sexual	2011
228	GUCC5205	Yamanashi	YN	Soil of strawberry	Sexual	2011
229	GUCC 5124	Yamanashi		*Erica formosa*	Sexual	2009
230	GUCC 5125	Yamanashi		*Erica formosa*	Sexual	2009
231	GUCC 5126	Yamanashi		*Erica formosa*	Sexual	2009
232	YnSIL5sh	Yamanashi		Strawberry	Sexual	2009

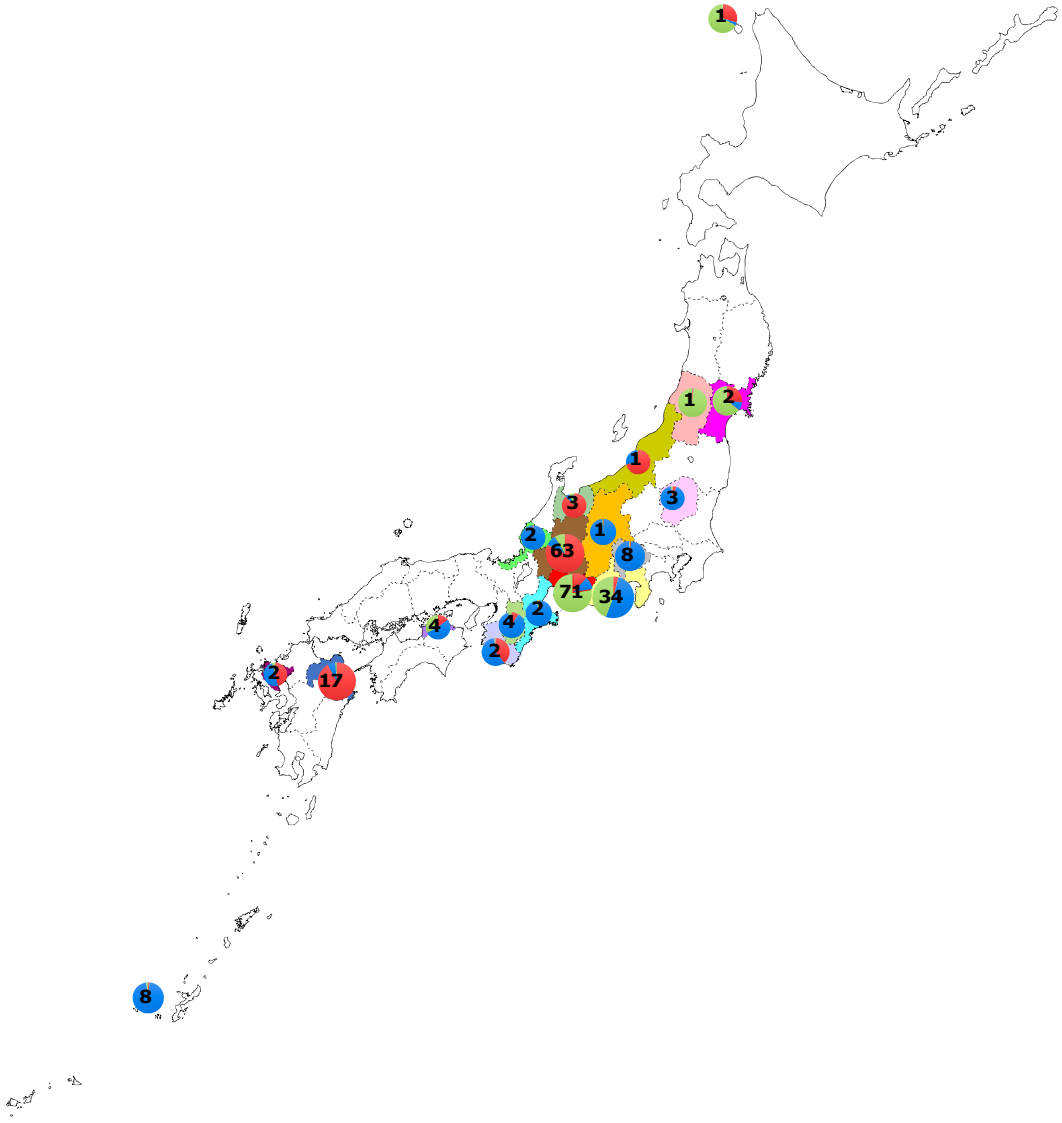

Figure 7. Prefectures in Japan where *P. helicoides* isolates were collected, with numbers of isolates collected. Pie diagrams indicate the genetic clustering of each population based on the STRUCTURE analysis. ■ Aichi; ■ Fukui; ■ Gifu; □ Hokkaido ■ Iriomote; ■Kagawa; ■ Mie; ■ Miyagi; ■ Nagano; ■ Nara; ■ Niigata ■Oita; ■ Saga; ■Shizuoka; ■ Tochigi; ■ Toyama; ■ Wakayama ■ Yamagata ■ Yamanashi

DNA was extracted using the PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems). First, each isolate was cultured on V8 medium to increase the number of mycelia produced. The mycelium was harvested by directly transferring it to 200 ml reagent-water suspension (1:1) and DNA was extracted using the manufacturer's protocol.

Microsatellite marker development and PCR reactions

The microsatellite markers were developed using the suppression PCR and thermal asymmetric interlaced (TAIL)-PCR methods, as described by Yin-Ling et al. (2009). Briefly, the suppression step was performed as follows: Genomic DNA of isolate GUCC 5015 was digested with AluI (Toyobo), then the fragments were purified and ligated to two adaptors: a 48-mer (5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3') and an 8-mer with the 3' end capped by an amino residue (5'-ACCAGCCC-NH_2-3'). The resulting DNA library was then used as the template in PCR amplifications using the adapter primer AP2 (5'-CTATAGGGCACGCGTGGT-3') and one of 6 microsatellite primers: $(AGC)_{10}$, $(CAA)_{10}$, $(CTT)_{10}$, $(GGA)_{10}$, $(TCA)_{10}$, and $(TGC)_{10}$. The PCR products were cloned and used to transform ECOS™ JM109 competent cells (Nippongene). After culturing, individual colonies were picked and used directly in PCR reactions with the M13M4 and M13Rv primers to amplify the cloned DNA fragments. The cloned fragments were sequenced using the TOPO® TA cloning® kit (Invitrogen). TAIL-PCR was then used to develop primers which would amplify the flanking regions of the microsatellite markers. Three sense primers (a, b, and c) were designed for each sequence

obtained in the suppression step (S1 Table). The sense primers were used in 3 consecutive PCR reactions (c, b, then a) with the arbitrary degenerate primer AD4 (5'-gtNcgaSWcaNaWgtt-3')(Liu and Whittier 1995) that binds to various places in the genome. The first PCR was performed in 25 μl with 20 ng genomic GUCC 5015 DNA, 0.2 μM primer c, 5 μM AD4 primer, 0.5 Units TaKaRa Taq DNA polymerase (Takara Bio Inc.), 0.2 mM dNTPs, and 1 × PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$). The second PCR was performed in 25 μl with 1 μl of a 50-fold dilution of the products of the first reaction, 0.2 μM primer b, 3 μM AD4 primer, 0.5 Units TaKaRa Taq DNA polymerase, 0.2 mM dNTPs, and 1 × PCR buffer. The third PCR was performed in 50 μl with 1 μl of a 50-fold dilution of the products of the second reaction, 0.2 μM primer a, 0.2 μM AD4 primer, 3.5 Units TaKaRa Taq DNA polymerase (Takara Bio Inc.), 0.2 mM dNTPs, and 1 × PCR buffer.

Major bands from the TAIL-PCR reactions were sequenced as described above and used to design specific PCR primers that flanked the microsatellites using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). These and 5 primer sets from a previous study [13] were tested with 3 strains of *P. helicoides* (GUCC 5056, GUCC 5076, and GUCC 5135). PCR reactions were carried out, and the products were cloned and sequenced using standard procedures.

Six primer pairs were selected for PCR analysis of all 232 *P. helicoides* isolates. Reactions were performed in a total volume of 25 μl containing 2 μL of 1 ng/μL DNA, 2.5 μL of 10 × PCR Buffer (plus Magnesium, Takara), 2.5 μl of 4 mg/mL BSA, 2.5 μL each of 10 mM forward and reverse primers, 2 μL of 2.5 mM dNTP mix (Takara), 0.1 μL rTaq polymerase (Takara), and 10.9 μL H_2O. The following conditions were used: 94ºC

for 5 min; 35 cycles of 94ºC for 30 s, 50 or 60 ºC for 30 s, and 72 ºC for 30 s; then 72 ºC for 7 min. The products were assessed by GelRed staining (Biotium) using 2% agarose gels in 0.5 × Tris-Acetate-EDTA buffer, and bands were visualized under UV light. The fragments were analyzed on an ABI3100 or ABI3130 Genetic Analyzer (Applied Biosystem) using the LIZ 250 DNA ladder as a marker. The electropherograms were scored manually.

Locus characteristics and diversity analysis

The numbers of alleles, numbers of unique alleles, and observed and expected heterozygosities were calculated using GenAlex v 6.503 (Peakall and Smouse 2006, 2012). The analysis of molecular variance as well as the fixation index was investigated using the same program with 9999 permutations. Populations that consisted of only one sample were eliminated to make the calculation possible.

Population structure analysis

Population structures were analyzed by cluster analysis, which was performed by estimating the probabilities of genotypes being distributed into K clusters using STRUCTURE v. 2.3.4. (Pritchard, Stephens, and Donnelly 2000; Falush, Stephens, and Pritchard 2003, 2007; Hubisz et al. 2009). Nine independent runs were performed for each K = 1–20. Each run assumed population admixture for correlated allele frequencies with 200000 burn-in lengths followed by 100000 repetitions of Markov chain Monte Carlo (MCMC). The optimal number for K was determined using STRUCTURE

HARVESTER (Earl and vonHoldt 2012) and matched up from the independent runs using CLUMPP (Jakobsson and Rosenberg 2007). The result was finally visualized using DISTRUCT (Rosenberg 2004).

Individual genetic distances were calculated using GenAlex (Peakall and Smouse 2006, 2012) and the phylogenetic tree was constructed using MEGA V. 6.0(Tamura et al. 2013) with the neighbor-joining algorithm. Individuals on the phylogenetic tree were assigned colors and shapes based on geographic (prefecture) and host origins, respectively, for easier interpretation.

Results

Development of microsatellite markers and diversity analysis of *P. helicoides*

We used a combination of suppression PCR and thermal asymmetric interlaced (TAIL)-PCR (Liu and Whittier 1995) to develop a set of microsatellite markers for genetic analyses of *P. helicoides*. For the suppression PCR step we made a genomic DNA library from *P. helicoides* isolate GUCC 5015. The genomic DNA was digested with a restriction enzyme and ligated with two adaptors, a 48-mer and an 8-mer. The 8-mer was capped with an amino residue at its 3' end and was complementary to one end of the 48-mer. This library was used in multiple PCR reactions, each using the AP2 primer, which identical to part of the 48-mer, and one of 6 microsatellite primers that were complementary to various simple DNA repeats. Amplification of fragments with the same adaptor at each end was suppressed because of intramolecular hybridization between the two adaptors. In the first PCR cycle, DNA elongation occurred only where a

microsatellite primer annealed to its complementary sequence in a genomic DNA fragment. In subsequent cycles the region between the AP2 primer and the microsatellite primer was amplified (Lian and Hogetsu 2002).

The suppression PCR products were cloned, and 41 individual colonies were picked and sequenced to check for the presence of the microsatellite regions. We then used the sequences to develop 41primer sets (S1 Table), which we paired with an arbitrary degenerate primer (AD4) for TAIL-PCR (Liu and Whittier 1995). Each primer set consisted of 3 sense primers (a, b, and c) that were used in 3 consecutive PCR reactions. The first reaction was performed using genomic DNA from isolate GUCC 5015 as the template; the second used the products of the first reaction as the template; and the third used the products of the second reaction as the template. This process can result in the highly specific amplification of PCR products containing the target microsatellite sequences. We obtained amplified products from 27 of the primer sets. After sequencing we found that 7 primer sets amplified microsatellite regions, and that two additional motifs, (AGGCA)n and (GCAGAC)n, were observed among the regions. We used those 7 amplicons to design specific PCR primers that flanked the microsatellites. These primers along with 5 primer pairs from a previous study (Yin-Ling et al. 2009)[13] were used in PCR reactions with 3 strains of *P. helicoides* (GUCC 5056, GUCC 5076, and GUCC 5135) as templates (Table 6). The PCR products were cloned and sequenced. Some primers amplified more than 2 alleles from a single isolate and would not be useful in population genetics studies. Two primer pairs amplified monomorphic regions from all three isolates, and these were also discarded. We identified 6 primer pairs that were suitable for population genetics analyses. The sequences of these primer pairs are shown in Table 7.

Table 6. Selection of microsatellite markers used in this study

No	Primer	Amplicon size		
		GUCC5056	**GUCC5076**	**GUCC5135**
1	YL-AG[a]	117, 119, 121	117, 119, 121, 123	117, 119, 121
2	YL-AGC[a]	285	285	285
3	YL-CAA[a]	519	519	519
4	YL-CTTT[a]	151, 171, 175, 179, 183, 187, 191, 195, 199, 203, 215	151, 155, 163, 167, 171, 175, 179, 183, 187, 191, 195, 199, 203, 207, 215	147, 163, 167, 171, 175, 179, 183, 187, 191, 199
5	YL-TCA[a]	166, 184, 187	175, 190	187
6	EM-AGC1	71, 77	71, 80	71,77
7	EM-CTT2	126, 141	117, 138, 144	111, 114, 117, 123, 141, 144
8	EM-GGA1	77, 80	86	77, 86
9	EM-GGA2	126, 138	138	68
10	EM-AGGCA1	78,88	94	100
11	EM-CTT1	55, 70	55, 70	70
12	EM-GCAGAC	94	94	76,94

[a]: Yin-Ling, et al., 2009

We analyzed all 232 isolates of *P. helicoides* using the selected primer pairs, which amplified microsatellites at 6 loci (Table 7). In total, 90 alleles were observed, indicated a high degree of allelic diversity at these loci. Four of the 6 loci showed significantly lower levels of observed heterozygosity than expected. This was due low rates of recombination at the loci even though each locus had many alleles.

Table 7. Summary of genetic variation and fixation index from each selected microsatellite locus

Locus	Microsatellite motif	Primer sequence(5`-3`)	Tm(°C)[a]	No.of alleles	No.of unique alleles	Ho[b]	He[c]	Fst[d]
EM-CTT1	(CTT)n	GCATTTCCAAGAGGAACCCGCC ATGGGGCAAGTCCAGCCCAAAAG	50	10	4	0.771	0.594	0.085
EM-AGC1	(AGC)n	CCGAGTCTACACCAACATGTTCACC TGCGTCTGCATCTGTGCGTG	60	17	1	0.719	0.805	0.055
EM-GCAGAC	(GCAGAC)n	ACCTCGGTGACAGCAGTGATC AGGCTTCTGCGGTGTCTACG	60	13	4	0.387	0.557	0.119
EM-GGA1	(GGA)n	AGCAGGGTTTGTTGCTGGAAG ACGATCCCTCCGCCATATCC	60	21	9	0.531	0.739	0.145
EM-GGA2	(GGA)n	GTGACGAGAATTCGAGCGTGTG TGGTGGATGGATCTCTTCAACCTAC	60	13	6	0.411	0.753	0.333
EM-AGGCA	(AGGCA)n	CGAATGGATATCGGCACGCC TGGGTCTGCCAATGGGTCTG	60	16	3	0.811	0.802	0.064

[a]:Tm(°C): Annealing Temperature
[b]: observed heterozygosity
[c]: Expected heterozygosity
[d]: Fixation index

The analysis of molecular variance was done by assigning each isolate to a population based on geographic origin. This analysis revealed greater variance within populations (81%) than among populations (19%) (Table 8). The fixation index for each locus was between 0.055 and 0.333 (Table 3). These indicate low to moderate genetic

differentiation between populations, suggesting that gene flow occurs between populations.

Table 8. Summary of analysis of molecular variance

Source	**df**[a]	**SS**[b]	**MS**[c]	**Est. Var.**[d]	**%**[e]
Among Pops	15	184.574	12.305	0.435	19%
Within Pops	438	817.039	1.865	1.865	81%
Total	453	1001.612		2.300	100%
Among Cluster	2	102.334	51.167	0.322	14%
Within Cluster	461	926.070	2.009	2.009	86%
Total	463	1028.403		2.330	100%

[a]: degree of freedom
[b]: sum of square
[c]: mean of square
[d]: estimated variance
[e]: percent of variance

Population structure analysis

The population structure analysis was performed using the STRUCTURE software. STRUCTURE uses a model-based clustering method that can accurately cluster individuals into genetic groups by estimating different numbers of clusters (K). The STRUCTURE HARVESTER then processes the STRUCTURE results to determine the best K that fits with the data. CLUMPP is used to align the cluster assignments across replicate analyses and the results are then visualized by DISTRUCT[21]. The clustering analysis performed suggested that K=3 is the most likely scenario for all samples tested (Fig 8a). The K=3 scenario was applied to all 21 geographic populations. The results highlighted the divergence between the Gifu, Oita, and Toyama populations (mainly red) and the other populations (mainly green and/or blue) (Fig 8b). The Aichi population

consisted of all 3 genetic groups with green as the majority. The Shizuoka population had two genetics groups (green and blue) with blue as the majority. The Tochigi, Yamanashi, and Iriomote populations also clustered in the blue genetic group while the Kagawa, USA, and Yamagata populations belonged mainly to the red genetic group (Fig. 8b). Pie diagrams showing the distribution of each genetic cluster within the 19 prefectures in Japan also revealed the dominancy of a particular group in most prefectures (Fig. 7). Miyagi, Yamagata, Rebun Island, and Aichi were dominated by the green group; Gifu, Toyama, Niigata, and Oita were dominated by the red group; and other regions were dominated by the blue group except for Shizuoka, which had similar amounts of blue and green.

The clustering results were supported by the phylogenetic tree, which divided all the samples into three clades (Fig. 9). The individuals belonging to the red, blue, and green genetic groups in the STRUCTURE analysis were mainly clustered on the phylogenetic tree in the first, second, and third clades, respectively. On the tree, the prefecture origin of each isolate is indicated by color (Fig. 8) The first clade (red group) contained many isolates from Gifu and Oita, the second clade (blue) had many isolates from Shizuoka and Aichi, and the last clade (green) had many from Aichi.

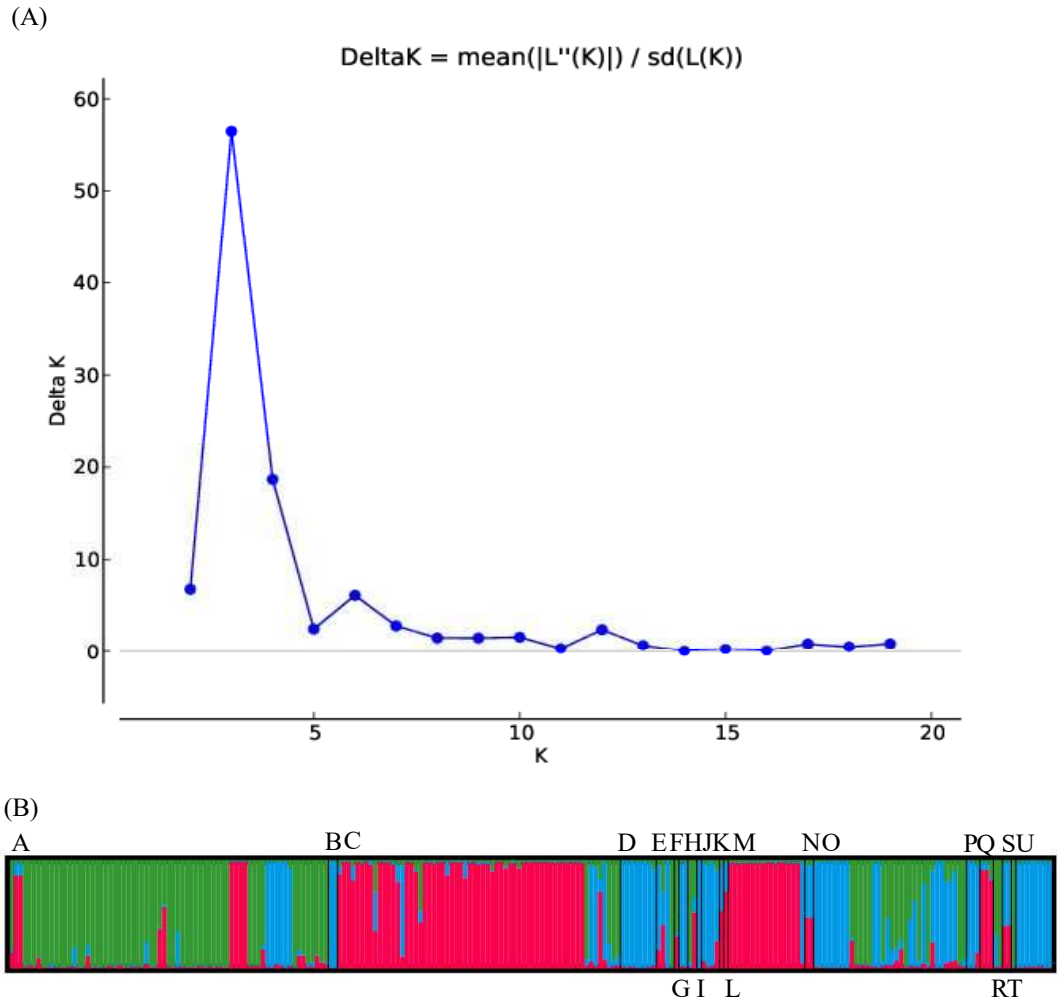

Figure 8. STRUCTURE analysis of 232 isolates of *P. helicoides*. (A) ΔK was calculated using STRUCTURE HARVESTER, and the results indicated that the most likely number of genetic clusters (K) was 3. (B) Histogram showing the estimated proportions of genetic clusters in each of 21 populations, based on K=3 clusters (red, green, and blue). A: Aichi; B: Fukui; C: Gifu; D: Okinawa; E: Kagawa; F: Hokkaido; G: Mie; H: Miyagi; I: Nagano; J: Nara; K: Netherland; L: Niigata; M: Oita; N: Saga; O: Shizuoka; P: Tochigi; Q: Toyama; R: USA; S: Wakayama: T: Yamagata; U: Yamanashi

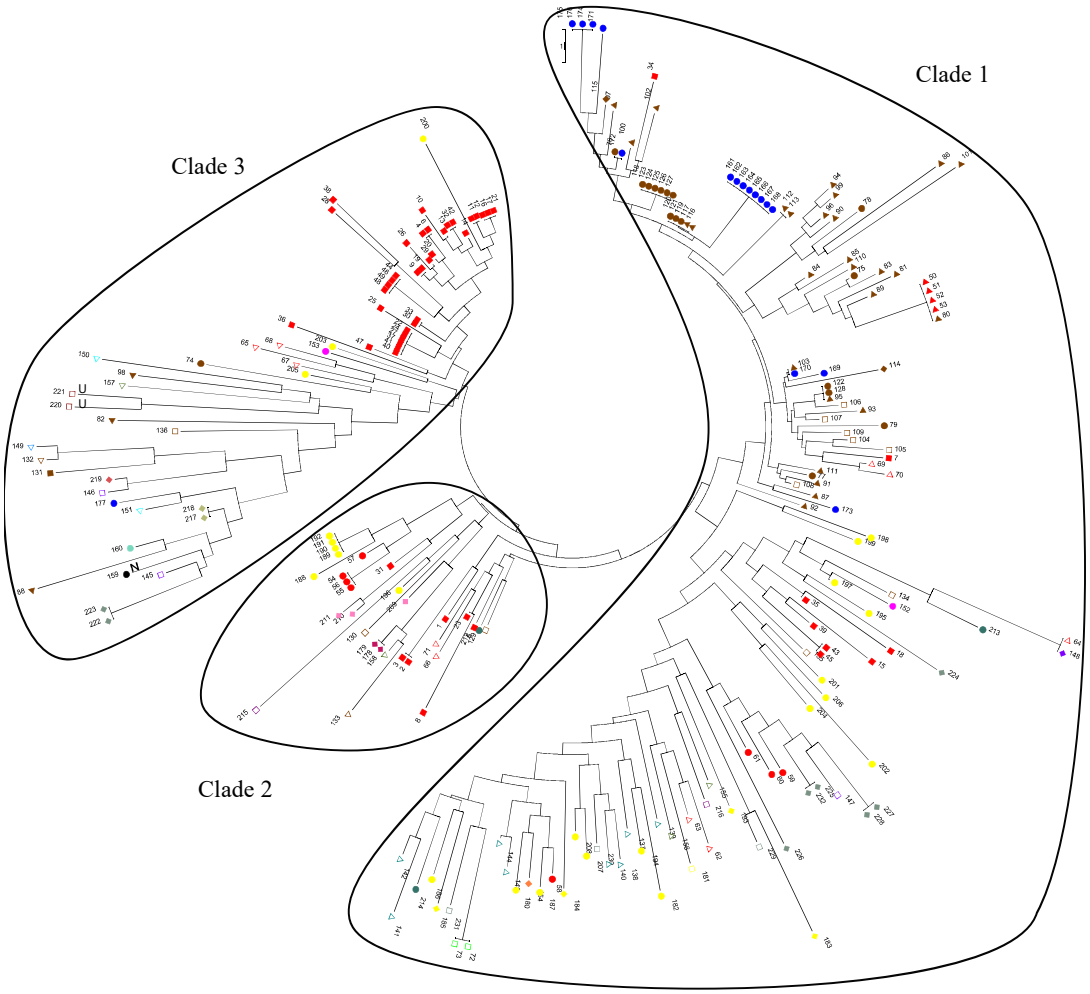

Figure 9. A phylogenetic tree of the *P. helicoides* populations in Japan. Hosts: ■ poinsettia; ▲ miniature rose; ● rose; ♦ strawberry; Δ natural environment; □ others. Geographic origin (by prefecture): ■ Aichi; ■ Fukui; ■ Gifu; □ Hokkaido ■ Iriomote; ■Kagawa; ■ Mie; ■ Miyagi; ■ Nagano; ■ Nara; ■ Niigata ■Oita; ■ Saga; ■Shizuoka; ■ Tochigi; ■ Toyama; ■ Wakayama ■ Yamagata ■ Yamanashi; N: Netherland; U: USA

Host origin is indicated by shape on the phylogenetic tree (Fig. 9). Isolates from rose and miniature rose tended to group together. In the first clade of the tree, isolates from cutting rose in Oita (isolate numbers 161–168) grouped with isolates from rose and miniature rose in Gifu. The isolates from miniature rose in Aichi (numbers 50–53) shared the same branch with an isolate from miniature rose in Gifu (number 80). In the second clade, isolates from rose in Shizuoka (numbers 188–192) grouped with isolates from rose in Aichi (numbers 54–57). Isolate 188 from Shizuoka and all the isolates from Aichi were asexual.

Unlike those from rose and miniature rose, the isolates from poinsettia in Aichi were distributed unevenly among the phylogenetic clades. The isolates from KZ farm (numbers 1–3; see Table 5) were in clade 2 whereas most isolates from TY farm were in clade 3 (numbers 4, 6, 7, 9–14, 16, 17, 19–22, 24–30, 32, 33, 36, and 37–42). Some isolates from TY farm were in clade 1 (numbers 18, 15, and 43–45) and 3 were clade 2 (numbers 2, 23, and 31).

The isolates from natural environments were also scattered among clades. In some cases, they were grouped with isolates from agricultural areas in the same geographic regions. For example, in clade 1 the isolates from Aichi water (numbers 69 and 70) were closely related to an isolate from poinsettia (number 7). In other cases, the isolates from natural environments grouped more closely with isolates from natural environments in different geographic regions.

Discussion

Knowledge about the genetic diversity of *P. helicoides* has been very limited. Our previous study uncovered intra-isolate variation in the rDNA ITS region [11]. Microsatellite markers have been used to characterize the population structures of *Phytophthora cinnamomi* (Engelbrecht, Duong, and Berg 2017), *Ph. infestans* (Stroud et al. 2016), *Ph. sojae* (Stewart et al. 2011), *Ph. nicotianae* (Biasi et al. 2016), and *Ph. ramorum* (Coats, Elliott, and Chastagner 2016). Studies on microsatellite markers for *P. helicoides* were initiated by Yin-Ling et al (2009), but in that study only 3 isolates were used to check the reliability of the primers.

It is very difficult to develop microsatellite markers if the complete genome sequence is not available. In this study we used suppression PCR and TAIL-PCR to develop 7 novel microsatellite markers, and 6 of these were suitable for population genetics analyses (Table 3). In total, ninety alleles were obtained from the 6 markers, indicating that the loci were highly polymorphic and suitable for population genetics studies. As a homothallic species, *P. helicoides* is generally assumed to perform sexual reproduction by self-fertilization. However, we found putatively heterozygous individuals within each population, suggesting that outcrossing could occur in *P. helicoides*. The fixation index (Fst) data (Table 3) also suggested the possibility of gene flow between populations, indicating that migration and outcrossing could play important roles in the development of genetic diversity among individuals of *P. helicoides*. This result is supported by the study on rDNA ITS diversity in *P. helicoides* (Kageyama et al. 2007). Outcrossing of homothallic species is also observed in *Pythium ultimum* (Francis

and Clair 1997), *Py. irregulare* (Harvey et al. 2000), and *Phytophthora sojae* (Whisson et al. 1994).

The clustering analysis performed using STRUCTURE and the phylogenetic analysis showed clear congruence; each genetic group from the STRUCTURE analysis formed a clade on the phylogenetic tree, with the same isolates. The first clade contained many isolates from rose and miniature rose in Gifu, Oita, and Aichi. The second clade contained several Aichi and Shizuoka isolates, and the last was dominated by isolates from poinsettia in Aichi. Studies of *Ph. infestans* in China (Y. et al. 2012) and *Ph. austrocedrae* in Argentina (Vélez et al. 2014)also found clustering based on geographical origin. In the STRUCTURE analysis, we assumed admixture in populations that had less than 70% of one genetic group, (Y. et al. 2012) suggesting that the Wakayama, Saga, and Nagano populations are admixed. These were formed by gene flow between two or more genetically distinct populations (Pfaff et al. 2001).

Several *P. helicoides* isolates used in this study are asexual and therefore classified as Group P (Kageyama et al. 2003). These isolates have the ability to produce abundant quantities of zoospores. In the STRUCTURE analysis, asexual isolates with the same geographical origin were allocated to the same genetic groups. This was supported by our previous study, in which Group P isolates showed different banding patterns than those of a sexual strain in an RFLP analysis of the rDNA ITS region (Kageyama et al. 2003).

Phytopythium helicoides from natural environments could be an important source of disease outbreaks in nearby farms. On the phylogenetic tree, several isolates from natural environments were grouped with isolates from nearby agricultural areas. The

isolates from irrigation water in Aichi were grouped with other Aichi isolates in both the STRUCTURE and phylogenetic analyses. Interestingly, a *P. helicoides* outbreak occurred on a farm downstream of a putative natural source in Aichi, but not on a closer farm. This suggested that the *P. helicoides* strain could have been carried to the farm by river. Other isolates from irrigation water in Aichi were closely related to isolates from Gifu. There are rivers that flow from Gifu to Aichi, so it is possible that this water-borne disease was carried to Aichi by river. Our data suggest that *P. helicoides* might be native to many areas, even though it has only recently caused disease outbreaks. This may be due to the increasing use of hydroponics culture, which favors the spread of *P. helicoides*. Another factor might be global warming, which would encourage the growth of this high temperature tolerant species.

This study indicated that the host plants could have a significant influence on the population genetics of *P. helicoides*. The isolates from cutting rose in Gifu and Oita showed closer relationships than isolates from miniature rose in Gifu (Fig 3). This may be due to differences in the cultivation systems of cutting rose and miniature rose. Cutting roses are grown in hydroponic culture systems, while miniature roses are grown in potting media. It is possible that the pathogen is transported between farms in the hydroponic nutrients for cutting rose or the potting mix for miniature rose.

In conclusion, we found that *P. helicoides* has high variance within individuals, indicating a high degree of heterozygosity and the ability for outcrossing. The migration of the pathogen could be facilitated naturally, in drainage systems, or by human activity in the transportation of agricultural materials.

GENERAL DISCUSSION AND CONCLUSION

Comparation of two different methods for developing microsatellite markers

Microsatellite markers are highly informative DNA markers used in population genetics research. (Addisalem et al. 2015). There were three different classes of markers containing microsatellite sequences, they are: (1) Simple Sequence Repeat (SSR) it usually generated by amplifying in a PCR reactions with the use of primers complementary to flanking region; (2) ISSR (inter-simple sequence repeats), based on the amplification of regions between inversely oriented closely spaced microsatellites; and (3) SAMPL (selective amplification of microsatellite polymorphic loci), which utilises AFLP (amplified fragment-length polymorphism) methodology, with one exception – for the second amplification, one of the starters is complementary to the microsatellite sequence (Rakoczy-Trojanowska and Bolibok 2004). Recent study showed that the SSR marker is the most suitable for analyzing population structure on the small spatial scale with more recent migration event (50-100 years) (Tsykun et al. 2017). However, there are major drawbacks in using microsatellite because they need to be isolated de novo from most species being examined for the first time (Wan et al. 2004). This due to the microsatellite usually found in non-coding region where the nucleotide substitution is higher than in the coding region (Zane, Bargelloni, and Patarnello 2002). Besides the variation in number of repeats, nucleotide substitutions within the repeats are observed between species when employing the same primer pair and make the marker become unreliable (Clisson, Lathuilliere, and Crouau-Roy 2000).

There were several methods on developing microsatellite markers for population genetics study. However, basically development of microsatellite markers could be devided into several stages: (i) prior knowledge of nucleotide sequences in which microsatellite occur; (ii) design of oligonucleotides (or primers) complementary to the regions flanking the microsatellite; (iii) validation of primers by PCR and electrophoresis of the product of the reaction, and (iv) detection of polymorphisms among individuals (Vieira et al. 2016). In this study, we used two different methods in developing microsatellite markers. For *P. nicotianae*, the whole genome sequence draft is available, so we used the whole genome sequence data to find the microsatellite sequence using Tandem Repeat Finder. As for the *Ph. helicoides*, the whole genome sequence were not available when we started the study, so the microsatellite regions were found by developing the genomic DNA library using suppression PCR followed with TAIL PCR. The PCR method to develop microsatellite markers is more time consuming compare to using the whole genome sequence data. However, the accuracy of developing the primer by PCR is better than primers developed from whole genome sequence data. This study were initially developed 12 primer sets for *P. nicotianae* and 8 primer sets for *Ph. helicoides*. From 12 primer sets developed for *P. nicotianae*, only 6 primer sets were suitable for population genetics study, while on *Ph. helicoides*, 6 out of 8 primer sets were suitable for the study. The TAIL PCR method provide better insight for the targeted microsatellite region (Anupam et al. 2005).

Different transmission mode of *Phytophthora nicotianae* and *Phytopythium helicoides*

There are several different aspects that contribute to the difference in transmission mode of a plant pathogen. They are the mating system, host specifity, ploidy, and the scale of dispersal (Grünwald et al. 2017). The two pathogens used in this study have their unique biological features that make them difference in their population genetics structure. *Phytophthora nicotianae* is heterothallic and has broad host range, while *Ph. helicoides* is homothallic and has smaller host range than *P. nicotianae.*

In this study, the *P. nicotianae* population were not structured either by geographic or host origin. Similar result also revealed on the analysis of global *P. nicotianae* collection using mithochondrial and nuclear DNA sequences (Mammella et al. 2013), and other wide host range pathogen such as *P. plurivora* (Schoebel et al. 2014). Meanwhile the population study on *P. nicotianae* by using single host on local scale revealed the association of genetic diversity with geographical collection (Li et al. 2017). This revealed that the broad host range of the pathogen and the worldwide spread of the pathogen would make the genetic diversity of *P. nicotianae* in a worldwide scale is hard to distinguished. However, focusing on one host plant in small area while studying the population genetics using more sensitive markers did helps in revealing the migration pattern and the origin of the pathogen in the local area.

Phytopythium helicoides has smaller host range and some isolates has loss its ability in generating sexual structure. This study shown that the host plants could have a significant influence on the population genetics of *Ph. helicoides*. The isolates from cutting rose in Gifu and Oita showed closer relationships than the isolates from miniature rose in Gifu despite the far distance between the two prefectures. This may be due to

differences in the cultivation systems of cutting rose and miniature rose. Cutting roses are grown in hydroponic culture systems, while miniature roses are grown in potting media. It is possible that the pathogen is transported between farms in the hydroponic nutrients for cutting rose or the potting mix for miniature rose.

Future contribution of population genetics study

Population genetics consider the origin, maintenance and spatiotemporal distribution of genetic variation of species under the influences of mutation, gene flow, recombination, drift and selection (Jiasui 2016). They will determine the evolutionary potential, or the pathogen's ability to rapidly adapt to new environments such as developing resistance against a fungicide or overcoming a resistance gene in the plant host (McDonald and Linde 2002).

Studying genotype with large number of neutral markers can be done to answer questions concerning demographic processes and genome-wide effects. The large number of markers will makes more robust inferences possible on genetic structure, gene or genotype flow, and mating systems. For example, population genetic study on 213 strains of *Fusarium graminearum* using microsatellite markers revealed that the populations in German field represented a high degree in sexual recombination with sufficient genetic diversity that allow the pathogen to adapt to change in local environment (Talas and McDonald 2015).

Understanding the population genetics could provide insight into the pathogen origin, the migration process, host tracking, host jumping, as well as its hybridization and chromosome rearrangement in the emergence of new pathogen (Grünwald, McDonald, and Milgroom 2016). Studies on population genetics of *P. infestans* and its closed

relatives, *P. ipomoeae* and *P. mirabilis* revealed the origin of the pathogen in central Mexico (Goss et al. 2014; Grünwald and Flier 2005).

In conclusion, better understanding of the population genetics will give better insight in the biology of the pathogen and help in improving the disease management strategy.

SUMMARY

A global trading and an innovation in plant breeding can lead to the wide distribution of genetically homogeneous crops. These factors, along with the tendency of farmers to cultivate the same cultivars over large areas, could accelerate a pathogen dispersal to a new area. In other words, a continuous cropping of a single cultivar will induce a global distribution of a pathogen. On the other hand, genetic variability in a pathogen will has a direct impact in a virulence, a fungicide tolerance and an infectious strategy of the pathogen, resulting in a quick response to a new cultivar and a fungicide application. Study on a population genetics will help a better understanding in genetic variability in the pathogen population and mechanisms in an occurrence of a new pathogenic strain and a fungicide tolerance strain and its transmission mode.

In this study, we used microsatellite markers to understand the population genetics study on *Phytophthora nicotianae* and *Phytopythium helicoides*. The different methods were used for developing the markers of *P. nicotianae* and *Ph. helicoides.* The availability of whole genome sequence data of *P. nicotianae* made the primer development easier, while in *Ph. helicoides* the suppression and TAIL PCR methods were used to create the genome library for developing the markers. The suppression and TAIL PCR method is costly and time consuming but provide a reliable sequences to develop the primers.

In *P. nicotianae*, six new microsatellites markers were developed, consisting of totally 39 alleles. One hundred thirty eight isolates, including 125 from Japan and 13 from overseas, were shown to be variable by phylogenetic analysis, even though they were collected from the same host and geographic origin. This suggests that there

is no geographic or host plant clustering. Population structure analysis also revealed a highly admixed population of *P. nicotianae* in Japan.

In *Ph. helicoides,* 6 novel microsatellite markers were developed with 90 alleles in the 232 isolates. The analysis of molecular variance suggested that *P. helicoides* had high variance within individuals and low fixation indices between populations. A phylogenetic analysis revealed that the isolates collected from the same hosts and/or geographic origins were often grouped together. Furthermore, the isolates from non-agricultural fields and river water were grouped into the same populations.

Phytophthora nicotianae and *Ph. helicoides* are biologically different in mating system, host range, as well as their scale of dispersal. *Phytophthora nicotianae* has broader host range, heterothallic, and wider scale of dispersal as its infected major agricultural commodities in the world. In the other hand, *Ph. helicoides* is homothallic and did not cause any serious disease outbreaks until 1996, despite being first isolated in 1940s. These different features in *P. nicotianae* and *Ph. helicoides* will reflect to the differences in the infectious strategies as well as the population structures. Because of the broad host range, *P. nicotianae* causes diseases in a number of crops and spread out to all over the world by human activities. On the other hand, *Ph. helicoides* strongly depends on host plants and geographical origins. The isolates in natural ecosystem could have established as a plant pathogen by the introduction of new crops from other countries and hydroponic culture systems, and global warming.

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my main advisor Prof. Koji Kageyama, Laboratory of Fungal Ecology, River Basin Research Center, Gifu University, for his patience and support in overcoming numerous obstacles I have been facing through my research. Besides my main advisor, I would like to thank my co-advisors: Prof. Haruhisa Suga, Life Science Research Center, Gifu University and Prof. Suzuki Katsumi, Department of Bioresources Sciences, Shizuoka University for their insightful comments and encouragement, but also for the hard question which incented me to widen my research from various perspectives.

I also would like to thank The Dean of United Graduate School of Agricultural Science, Gifu University, Prof. Masateru Senge and The Director of Biotechnology Research Center, Universitas Gadjah Mada, Prof. Siti Subandiyah to give me opportunity to join double degree program between UGSAS Gifu University and Universitas Gadjah Mada.

I would like to acknowledge Mr. Seiji Uematsu, Dr. Hideki Watanabe, Minoru Inada, Dr. Yuji Kajitani for providing the *P. nicotianae* isolates to be used in this study, and Dr. Hirokazu Fukui, Dr. Hideki Watanabe, Mr. Taichi Funakubo, Dr. Noriyuki Miyako, and Mr. Mikihiko Suzuki for providing some of the *Ph. helicoides* isolates.

My sincere thanks also goes to Ms. Otsubo Kayako and Ms. Ayaka Hieno for helping me not only in laboratory problems but also in adaptation to live in Japan. I thank my fellow labmates for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last three years. Last but not least, I would like to thank The Japanese Government for giving me scholarship and open new opportunity for me to pursue PhD in Japan.

LITERATURE CITED

Addisalem, A. B., G. Danny Esselink, F. Bongers, and M. J.M. Smulders. 2015. "Genomic Sequencing and Microsatellite Marker Development for Boswellia Papyrifera, an Economically Important but Threatened Tree Native to Dry Tropical Forests." *AoB PLANTS* 7 (1): 1–11. https://doi.org/10.1093/aobpla/plu086.

Anupam, DIXIT, J I N Ming-Hua, CHUNG Jong-Wook, Y U Jae-Woong, CHUNG Hun-Ki, M A Kyung-Ho, PARK Yong-Jin, and C H O Eun-Gi. 2005. "Development of Polymorphic Microsatellite Markers in Sesame (Sesamum Indicum L.)." *Molecular Ecology Notes* 5 (4): 736–38. https://doi.org/10.1111/j.1471-8286.2005.01048.x.

Bala, K, G P Robideau, C A Levesque, A W A M de Cock, Z Gloria Abad, A M Lodhi, Saleem Shahzad, Ghaffar A, and M D Coffey. 2010. "Phytopythium Abad, de Cock, Bala, Robideau, Lodhi & Lévesque, Gen. Nov. and Phytopythium Sindhum Lodhi, Shahzad & Lévesque, Sp. Nov." *Persoonia: Molecular Phylogeny and Evolution of Fungi* 24 (June 2010): 127. https://doi.org/10.3767/003158510X512748.

Barrès, B., J. Carlier, M. Seguin, C. Fenouillet, C. Cilas, and V. Ravigné. 2012. "Understanding the Recent Colonization History of a Plant Pathogenic Fungus Using Population Genetic Tools and Approximate Bayesian Computation." *Heredity* 109 (5): 269–79. https://doi.org/10.1038/hdy.2012.37.

Beakes, Gordon W, and Satoshi Sekimoto. 2009. "The Evolutionary Phylogeny of Oomycetes." In *Oomycete Genetics and Genomics*, edited by Kurt Lamour and Sophien Kamoun, 1–24.

Bebber, Daniel P., Timothy Holmes, and Sarah J. Gurr. 2014. "The Global Spread of

Crop Pests and Pathogens." *Global Ecology and Biogeography* 23 (12): 1398–1407. https://doi.org/10.1111/geb.12214.

Benson, Gary. 1999. "Tandem Repeats Finder:A Program to Analyze DNA Sequences." *Nucleic Acids Res* 27 (2): 573–80. https://doi.org/10.1093/nar/27.2.573.

Bertier, Lien, Leen Leus, Liesbet D'hondt, Arthur W.A.M. De Cock, and Monica Höfte. 2013. "Host Adaptation and Speciation through Hybridization and Polyploidy in Phytophthora." *PLoS ONE* 8 (12): 1–15. https://doi.org/10.1371/journal.pone.0085385.

Biasi, Antonio, Frank N Martin, Santa O Cacciola, Gaetano Magnano di San Lio, Niklaus J. Grünwald, and Leonardo Schena. 2016. "Genetic Analysis of Phytophthora Nicotianae Populations from Different Hosts Using Microsatellite Markers." *Phytopathology* 106 (9): 1006–14. https://doi.org/10.1094/PHYTO-11-15-0299-R.

Bonnet, Ph, I. Lacourt, P. Venard, and P. Ricci. 1994. "Diversity in Pathogenicity to Tobacco and in Elicitin Production among Isolates of Phytophthora Parasitica." *Journal of Phytopathology* 141 (1): 25–37. https://doi.org/10.1111/j.1439-0434.1994.tb01442.x.

Brurberg, May Bente, Abdelhameed Elameen, Vinh Hong Le, Ragnhild Nærstad, Arne Hermansen, Ari Lehtinen, Asko Hannukkala, et al. 2011. "Genetic Analysis of Phytophthora Infestans Populations in the Nordic European Countries Reveals High Genetic Variability." *Fungal Biology* 115 (4–5): 335–42. https://doi.org/10.1016/j.funbio.2011.01.003.

Charlesworth, Brian. 2015. "What Use Is Population Genetics?" *Genetics* 200 (3): 667–69. https://doi.org/10.1534/genetics.115.178426.

Chellemi, D. O., D. J. Mitchell, M. E. Kannwischer-Mitchell, P. A. Rayside, and E. N.

Rosskopf. 2000. “Pythium Spp. Associated with Bell Pepper Production in Florida.” *Plant Disease* 84 (12): 1271–74. https://doi.org/10.1094/PDIS.2000.84.12.1271.

Chen, Lei, Shusheng Zhu, Xiaohong Lu, Zhili Pang, Meng Cai, and Xili Liu. 2012. “Assessing the Risk That Phytophthora Melonis Can Develop a Point Mutation (V1109L) in CesA3 Conferring Resistance to Carboxylic Acid Amide Fungicides.” *PLoS ONE* 7 (7). https://doi.org/10.1371/journal.pone.0042069.

Chen, Xiao Ren, Bei Bei Liu, Yu Ping Xing, Bao Ping Cheng, Mei Ling Liu, Yun Hui Tong, and Jing You Xu. 2016. “Identification and Characterization of Phytopythium Helicoides Causing Stem Rot of Shatangju Mandarin Seedlings in China.” *European Journal of Plant Pathology* 146 (4): 715–27. https://doi.org/10.1007/s10658-016-0952-4.

Cline, Erica T., David F. Farr, and Amy Y. Rossman. 2007. “A Synopsis of Phytophthora with Accurate Scientific Names, Host Range, and Geographic Distribution.” *Plant Health Progress* 10 (March): 61–62. https://doi.org/10.1094/PHP.

Clisson, I., M. Lathuilliere, and B. Crouau-Roy. 2000. “Conservation and Evolution of Microsatellite Loci in Primate Taxa.” *American Journal of Primatology* 50 (3): 205–14. https://doi.org/10.1002/(SICI)1098-2345(200003)50:3<205::AID-AJP3>3.0.CO;2-Y.

Coats, Katie, Marianne Elliott, and Gary Chastagner. 2016. “Microsatellite Analysis of the EU1 Lineage of Phytophthora Ramorum in Washington State Nurseries , Landscapes , and Waterways 1,” 98371.

Cock, A W A M de, A M Lodhi, T L Rintoul, K Bala, G P Robideau, Z Gloria Abad, M D Coffey, S Shahzad, and C A Lévesque. 2015. “Phytopythium: Molecular Phylogeny and Systematics.” *Persoonia* 34: 25–39.

https://doi.org/10.3767/003158515X685382.

Colas, V, I Lacourt, P Ricci, F Vanlerberghe-Masutti, A Poupet, and F Panabières. 1998. "Diversity of Virulence in Phytophthora Parasitica on Tobacco, as Reflected by Nuclear RFLPs." *Phytopathology* 88 (3): 205–12. https://doi.org/10.1094/PHYTO.1998.88.3.205.

Derevnina, Lida, Benjamin Petre, Ronny Kellner, Yasin F. Dagdas, Mohammad Nasif Sarowar, Artemis Giannakopoulou, Juan Carlos De la Concepcion, et al. 2016. "Emerging Oomycete Threats to Plants and Animals." *Philosophical Transactions of the Royal Society B: Biological Sciences* 371 (1709): 20150459. https://doi.org/10.1098/rstb.2015.0459.

Dobrowolski, M. P., I. C. Tommerup, B. L. Shearer, and P. A. O'Brien. 2003. "Three Clonal Lineages of *Phytophthora Cinnamomi* in Australia Revealed by Microsatellites." *Phytopathology* 93 (6): 695–704. https://doi.org/10.1094/PHYTO.2003.93.6.695.

Drenth A. et al. 1993. "Genotypic Diversity of Phytophthora Infestans in The Netherlands Revelead by DNA Polymorphisms." *Phytopathology*.

Drenth, A., and Stephen B Goodwin. 1999. "Chapter 9 Population Structure of Oomycetes." In *Structure and Dynamics of Fungal Populations*, edited by James J Worrall, 195–224. Springer Science+Business Media.

Drenth, A, S C Whisson, D J Maclean, J A G Irwin, N R Obst, and M J Ryley. 1996. "The Evolution of Races of Phytophthora Sojae in Australia." *Phytopathology*. https://doi.org/10.1094/Phyto-86-163.

Earl, Dent A., and Bridgett M. vonHoldt. 2012. "STRUCTURE HARVESTER: A Website and Program for Visualizing STRUCTURE Output and Implementing the

Evanno Method." *Conservation Genetics Resources* 4 (2): 359–61. https://doi.org/10.1007/s12686-011-9548-7.

Engelbrecht, J., T. A. Duong, and N. V.D. Berg. 2017. "New Microsatellite Markers for Population Studies of Phytophthora Cinnamomi, an Important Global Pathogen." *Scientific Reports* 7 (1): 1–10. https://doi.org/10.1038/s41598-017-17799-9.

Falush, Daniel, Matthew Stephens, and Jonathan K. Pritchard. 2003. "Inference of Population Structure Using Multilocus Genotype Data: Linked Loci and Correlated Allele Frequencies." *Genetics* 164 (4): 1567–87. https://doi.org/10.1111/j.1471-8286.2007.01758.x.

———. 2007. "Inference of Population Structure Using Multilocus Genotype Data: Dominant Markers and Null Alleles." *Molecular Ecology Notes* 7 (4): 574–78. https://doi.org/10.1111/j.1471-8286.2007.01758.x.

Forbes, G a, X C Escobar, C C Ayala, J Revelo, M E Ordoñez, B a Fry, K Doucett, and W E Fry. 1997. "Population Genetic Structure of Phytophthora Infestans in Ecuador." *Phytopathology* 87 (4): 375–80. https://doi.org/10.1094/PHYTO.1997.87.4.375.

Förster, H, BM Tyler, and MD Coffey. 1994. "Phytophthora Sojae Races Have Arisen by Clonal Evolution and by Rare Outcrosses." *Molecular Plant–Microbe Interactions*. https://doi.org/10.1094/MPMI-7-0780.

Francis, David M, and Dina A St. Clair. 1997. "Population Genetics of Pythium Ultimum." *Phytopathology* 87 (4): 454–61. https://doi.org/10.1094/PHYTO.1997.87.4.454.

Gisi, U, and Y Cohen. 1996. "Resistance to Phenylamide Fungicides: A Case Study with *Phytophthora Infestans* Involving Mating Type and Race Structure." *Annual Review of Phytopathology* 34: 549–72. https://doi.org/10.1146/annurev.phyto.34.1.549.

Goodwin, Stephen B. 1997. "Population Genetics of Soilborne Fungal Plant Pathogens:

The Population Genetics of *Phytophthora*." *Phytopathology* 87 (4): 462–73. https://doi.org/10.1094/PHYTO.1997.87.4.448.

Goss, E. M., J. F. Tabima, D. E. L. Cooke, S. Restrepo, W. E. Fry, G. A. Forbes, V. J. Fieland, M. Cardenas, and N. J. Grunwald. 2014. "The Irish Potato Famine Pathogen Phytophthora Infestans Originated in Central Mexico Rather than the Andes." *Proceedings of the National Academy of Sciences* 111 (24): 8791–96. https://doi.org/10.1073/pnas.1401884111.

Grünwald, Niklaus J., and W E Fry. 2010. "Introduction to Oomycetes." The Plant Health Instructor. 2010. https://doi.org/10.1094/PHI-I-2010-1207-01.

Grünwald, Niklaus J., and Erica M. Goss. 2011. "Evolution and Population Genetics of Exotic and Re-Emerging Pathogens: Novel Tools and Approaches." *Annual Review of Phytopathology* 49 (1): 249–67. https://doi.org/10.1146/annurev-phyto-072910-095246.

Grünwald, Niklaus J., Bruce A. McDonald, and Michael G. Milgroom. 2016. "Population Genomics of Fungal and Oomycete Pathogens." *Annual Review of Phytopathology* 54 (1): 323–46. https://doi.org/10.1146/annurev-phyto-080614-115913.

Grünwald, Niklaus J, Sydney E Everhart, B J Knaus, Zhian N Kamvar, N J Grünwald, S E Everhart, and Z N Kamvar. 2017. "Best Practices for Population Genetic Analyses." *Papers in Plant Pathology* 107 (9): 1000–1010. https://doi.org/10.1094/PHYTO-12-16-0425-RVW.

Grünwald, Niklaus J, and Wilbert G Flier. 2005. "The Biology of Phytophthora Infestans at Its Center of Origin." *Annual Review of Phytopathology* 43 (1): 171–90. https://doi.org/10.1146/annurev.phyto.43.040204.135906.

Han, Kyung Sook, Seung Beom Hong, Seong Chan Lee, You Kyoung Han, and Dae Ho

Kim. 2010. “Root Rot of Rose Caused by Pythium Helicoides in Korea TT - Root Rot of Rose Caused by Pythium Helicoides in Korea.” *The Plant Pathology Journal* 26 (4): 429. http://kiss.kstudy.com/search/detail_page.asp?key=2953383.

Harvey, P. R., P. J. Butterworth, B. G. Hawke, and C. E. Pankhurst. 2000. “Genetic Variation among Populations of *Pythium Irregulare* in Southern Australia.” *Plant Pathology* 49 (5): 619–27. https://doi.org/10.1046/j.1365-3059.2000.00493.x.

Horie, Hiromichi. 2007. “Studies on Diagnosis, Ecology, and Control of Plant Diseases on Various Horticultural Crops in Japan.” *Journal of General Plant Pathology* 73 (6): 414–17. https://doi.org/10.1007/s10327-007-0053-5.

Huang, Jing, Na Guo, Yinghui Li, Jutao Sun, Guanjun Hu, Haipeng Zhang, Yanfei Li, et al. 2016. “Phenotypic Evaluation and Genetic Dissection of Resistance to Phytophthora Sojae in the Chinese Soybean Mini Core Collection.” *BMC Genetics* 17 (1): 1–14. https://doi.org/10.1186/s12863-016-0383-4.

Hubisz, Melissa J., Daniel Falush, Matthew Stephens, and Jonathan K. Pritchard. 2009. “Inferring Weak Population Structure with the Assistance of Sample Group Information.” *Molecular Ecology Resources* 9 (5): 1322–32. https://doi.org/10.1111/j.1755-0998.2009.02591.x.

Ishiguro, Yasushi, Kayoko Otsubo, Hideki Watanabe, Mikihiko Suzuki, Kiichi Nakayama, Takashi Fukuda, Masashi Fujinaga, Haruhisa Suga, and Koji Kageyama. 2014. “Root and Crown Rot of Strawberry Caused by Pythium Helicoides and Its Distribution in Strawberry Production Areas of Japan.” *Journal of General Plant Pathology* 80 (5): 423–29. https://doi.org/10.1007/s10327-014-0520-8.

Ivors, Kelly L., Katherine J. Hayden, Peter J.M. Bonants, David M. Rizzo, and Matteo Garbelotto. 2004. “AFLP and Phylogenetic Analyses of North American and

European Populations of Phytophthora Ramorum." *Mycological Research* 108 (4): 378–92. https://doi.org/10.1017/S0953756204009827.

Jakobsson, Mattias, and Noah A. Rosenberg. 2007. "CLUMPP: A Cluster Matching and Permutation Program for Dealing with Label Switching and Multimodality in Analysis of Population Structure." *Bioinformatics* 23 (14): 1801–6. https://doi.org/10.1093/bioinformatics/btm233.

Jiasui, Zhan. 2016. "Population Genetics of Plant Pathogens." In *ELS*, 1–7. American Cancer Society. https://doi.org/10.1002/9780470015902.a0021269.pub2.

Judelson, Howard S. 2012. "Dynamics and Innovations within Oomycete Genomes: Insights into Biology, Pathology, and Evolution." *Eukaryotic Cell* 11 (11): 1304–12. https://doi.org/10.1128/EC.00155-12.

Kageyama, Koji, T. Aoyagi, R Sunouchi, and H. Fukui. 2002. "Root Rot of Miniature Roses Caused by Pythium Helicoides." *Journal of General Plant Pathology* 68 (1): 15–20. https://doi.org/10.1007/PL00013047.

Kageyama, Koji, Masako Senda, Takahiro Asano, Haruhisa Suga, and Kiyoshi Ishiguro. 2007. "Intra-Isolate Heterogeneity of the ITS Region of RDNA in Pythium Helicoides." *Mycological Research* 111 (4): 416–23. https://doi.org/10.1016/j.mycres.2007.01.019.

Kageyama, Koji, M. Suzuki, A. Priyatmojo, Y. Oto, K. Ishiguro, H. Suga, T. Aoyagi, and H. Fukui. 2003. "Characterization and Identification of Asexual Strains of Pythium Associated with Root Rot of Rose in Japan." *Journal of Phytopathology* 151 (9): 485–91. https://doi.org/10.1046/j.1439-0434.2003.00757.x.

Kanto, T., S. Uematsu, and M. Aino. 2007. "Phytophthora Blight of Poinsettia (Euphorbia Pulcherrima Willd. Ex Klotzsch.) Caused by Phytophthora Nicotianae van Breda de

Haan (1896)." *Japanese Journal of Phytopathology* 73 (2): 112–13. https://doi.org/10.3186/jjphytopath.73.112.

Keeling, Patrick J. 2009. "Chromalveolates and the Evolution of Plastids by Secondary Endosymbiosis." *Journal of Eukaryotic Microbiology* 56 (1): 1–8. https://doi.org/10.1111/j.1550-7408.2008.00371.x.

Koh, Y J, S B Goodwin, A T Dyer, B A Cohen, A Ogoshi, N Sato, and W E Fry. 1994. "Migrations and Displacements of Phytophthora Infestans Populations in East Asian Countries." *Phytopathology*.

Li, Yonggang, Karen Harris-Shultz, Hongliang Wang, Phil Wadl, and Pingsheng Ji. 2017. "Population Structure and Genetic Diversity of *Phytophthora Nicotianae* from Tobacco in Georgia." *Plant Disease*, no. March: PDIS-01-17-0142-RE. https://doi.org/10.1094/PDIS-01-17-0142-RE.

Lian, C, and T Hogetsu. 2002. "Development of Microsatellite Markers in Black Lotus (Robinia Pseudoacacia) Using a Dual-Supression-PCR Technique." *Molecular Ecology Notes* 2 (4): 618–20. https://doi.org/10.1046/j.1471-8278 .2002.00213.x.

Link, Virginia Heffer, Mary L Powelson, and Kenneth B Johnson. 2012. "Oomycetes." The Plant Health Instructor. 2012.

Liu, Yao-Guang, and Robert F. Whittier. 1995. "Thermal Asymmetric Interlaced PCR: Automatable Amplification and Sequencing of Insert End Fragments from P1 and YAC Clones for Chromosome Walking." *Genomics* 25 (3): 674–81. https://doi.org/10.1016/0888-7543(95)80010-J.

Ma, Liang, Ya Jie Ji, and De Xing Zhang. 2015. "Statistical Measures of Genetic Differentiation of Populations: Rationales, History and Current States." *Current Zoology* 61 (5): 886–96. https://doi.org/10.1093/czoolo/61.5.886.

Mammella, Marco a, Frank N Martin, Santa O Cacciola, Michael D Coffey, Roberto Faedda, and Leonardo Schena. 2013. “Analyses of the Population Structure in a Global Collection of Phytophthora Nicotianae Isolates Inferred from Mitochondrial and Nuclear DNA Sequences.” *Phytopathology* 103 (6): 610–22. https://doi.org/10.1094/PHYTO-10-12-0263-R.

McDonald, Bruce A., and Celeste Linde. 2002. “PATHOGEN POPULATION GeNETICS, EvOLUTIONARY POTENTIAL, AND DuraBLE RESISTANCE.” *Annual Review of Phytopathology* 40 (1): 349–79. https://doi.org/10.1146/annurev.phyto.40.120501.101443.

Montarry, Josselin, Didier Andrivon, Isabelle Glais, Roselyne Corbiere, Gladys Mialdea, and Francois Delmotte. 2010. “Microsatellite Markers Reveal Two Admixed Genetic Groups and an Ongoing Displacement within the French Population of the Invasive Plant Pathogen Phytophthora Infestans.” *Molecular Ecology* 19 (9): 1965–77. https://doi.org/10.1111/j.1365-294X.2010.04619.x.

Nakamura, H, and M Matsuzaki. 1994. “Occurance of Phytophthora Rot of Limonium Caused by Phytophthora Nicotianae in Saga Prefecture.” *Annals of the Phytopathological Society of Japan* 60 (6). https://ci.nii.ac.jp/els/contents110002737296.pdf?id=ART0003028115.

Nath, Vishnu Sukumari, Muthukrishnan Senthil, Vinayaka Mahabaleswar Hegde, Muthulekshmi Lajapathy Jeeva, Raj Shekhar Misra, Syamala Swayamvaran Veena, and Mithun Raj. 2013. “Genetic Diversity of Phytophthora Colocasiae Isolates in India Based on AFLP Analysis.” *3 Biotech* 3 (4): 297–305. https://doi.org/10.1007/s13205-012-0101-5.

Panabières, F, G S Ali, M B Allagui, R J D Dalio, N C Gudmestad, M Kuhn, S Guha Roy,

Leonardo Schena, and A Zampounis. 2016. “Phytophthora Nicotianae Diseases Worldwide: New Knowledge of a Long-Recognised Pathogen.” *Phytopathologia Mediterranea* 55 (1): 20–40. https://doi.org/10.14601/Phytopathol_Mediterr-16423.

Pang, Zhili, Jingpeng Shao, Lei Chen, Xiaohong Lu, Jian Hu, Zhaohai Qin, and Xili Liu. 2013. “Resistance to the Novel Fungicide Pyrimorph in Phytophthora Capsici: Risk Assessment and Detection of Point Mutations in CesA3 That Confer Resistance.” *PLoS ONE* 8 (2). https://doi.org/10.1371/journal.pone.0056513.

Parkunan, V, C S Johnson, B C Bowman, Virginia Tech, Southern Piedmont, C X Hong, Hampton Roads, Virginia Beach, and North Carolina. 2010. “Population Structure , Mating Type , and Mefenoxam Sensitivity of Phytophthora Nicotianae in Virginia Tobacco Fields.” *Plant Disease* 94 (11): 1361–65. https://doi.org/10.1094/PDIS-05-10-0338.

Peakall, Rod, and Peter E. Smouse. 2006. “GENALEX 6: Genetic Analysis in Excel. Population Genetic Software for Teaching and Research.” *Molecular Ecology Notes* 6 (1): 288–95. https://doi.org/10.1111/j.1471-8286.2005.01155.x.

———. 2012. “GenALEx 6.5: Genetic Analysis in Excel. Population Genetic Software for Teaching and Research-an Update.” *Bioinformatics* 28 (19): 2537–39. https://doi.org/10.1093/bioinformatics/bts460.

Pfaff, C.L., E.J. Parra, C. Bonilla, K. Hiester, P.M. McKeigue, M.I. Kamboh, R.G. Hutchinson, R.E. Ferrell, E. Boerwinkle, and M.D. Shriver. 2001. “Population Structure in Admixed Populations: Effect of Admixture Dynamics on the Pattern of Linkage Disequilibrium.” *The American Journal of Human Genetics* 68 (1): 198–207. https://doi.org/10.1086/316935.

Pritchard, Jonathan K., Matthew Stephens, and Peter Donnelly. 2000. “Inference of

Population Structure Using Multilocus Genotype Data." *Genetics* 155 (2): 945–59. https://doi.org/10.1111/j.1471-8286.2007.01758.x.

Rakoczy-Trojanowska, Monika, and Hanna Bolibok. 2004. "Characteristics and a Comparison of Three Classes of Microsatellite-Based Markers and Their Application in Plants." *Cellular & Molecular Biology Letters* 9 (2): 221–38. https://doi.org/10.1038/sj.embor.7400854.

Robideau, G P, A W A M de Cock, M D Coffey, H Voglmayr, H Brouwer, K Bala, D W Chitty, et al. 2011. "DNA Barcoding of Oomycetes with Cytochrome c Oxidase Subunit I and Internal Transcribed Spacer." *Molecular Ecology Resources* 11 (6): 1002–11. https://doi.org/10.1111/j.1755-0998.2011.03041.x.

Rosenberg, Noah A. 2004. "DISTRUCT: A Program for the Graphical Display of Population Structure." *Molecular Ecology Notes* 4 (1): 137–38. https://doi.org/10.1046/j.1471-8286.2003.00566.x.

Sansome, E. 1977. "Polyploidy and Induced Gametangial Formation in British Isolates of Phytophthora Infestans." *Journal of General Microbiology* 99 (2): 311–16. https://doi.org/10.1099/00221287-99-2-311.

Schoebel, Corine N., Jane Stewart, Niklaus J. Gruenwald, Daniel Rigling, and Simone Prospero. 2014. "Population History and Pathways of Spread of the Plant Pathogen Phytophthora Plurivora." *PLoS ONE* 9 (1). https://doi.org/10.1371/journal.pone.0085368.

Selkoe, Kimberly A., and Robert J. Toonen. 2006. "Microsatellites for Ecologists: A Practical Guide to Using and Evaluating Microsatellite Markers." *Ecology Letters* 9 (5): 615–29. https://doi.org/10.1111/j.1461-0248.2006.00889.x.

Stewart, Silvina, Damitha Wickramasinghe, Anne E. Dorrance, and Alison E. Robertson.

2011. “Comparison of Three Microsatellite Analysis Methods for Detecting Genetic Diversity in Phytophthora Sojae (Stramenopila: Oomycete).” *Biotechnology Letters* 33 (11): 2217–23. https://doi.org/10.1007/s10529-011-0682-9.

Stroud, J. A., D. S. Shaw, M. D. Hale, and K. A. Steele. 2016. “SSR Assessment of Phytophthora Infestans Populations on Tomato and Potato in British Gardens Demonstrates High Diversity but No Evidence for Host Specialization.” *Plant Pathology* 65 (2): 334–41. https://doi.org/10.1111/ppa.12407.

Sunnucks, Paul. 2000. “Efficient Genetic Markers for Population Biology.” *Tree* 15 (5): 199–203. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2729459&tool=pmcentrez&rendertype=abstract.

Takeuchi, Jun, and Hiromichi Horie. 2000. “First Report of Phytophthora Rot of Garden Pea and Albuca Nelsonii in Japan.” *Annual Report of the Kanto-Tosan Plant Protection Society* 2000 (47): 45–48. https://doi.org/10.11337/ktpps1999.2000.45.

Takeuchi, Jun, Hiromichi Horie, and Koki Eimori. 2004. “First Report of Phytophthora Rot of New Zealand Spinach in Japan.” *Annual Report of the Kanto-Tosan Plant Protection Society* 51: 55–57. https://www.jstage.jst.go.jp/article/ktpps1999/2004/51/2004_51_55/_pdf/-char/en.

Takeuchi, Taeko, and Takeshi Suzuki. 2009. “Kenkyuhoukoku_2_p1.Pdf.” *Annual Research Bulletin of the Chiba Prefectural Agriculture and Forestry Research Center* 2: 1–6. https://www.pref.chiba.lg.jp/lab-nourin/nourin/kenkyuuhoukoku/documents/kenkyuhoukoku_2_p1.pdf.

Talas, Firas, and Bruce A. McDonald. 2015. “Genome-Wide Analysis of Fusarium Graminearum Field Populations Reveals Hotspots of Recombination.” *BMC*

Genomics 16 (1): 1–12. https://doi.org/10.1186/s12864-015-2166-0.

Tamura, Koichiro, Glen Stecher, Daniel Peterson, Alan Filipski, and Sudhir Kumar. 2013. "MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0." *Molecular Biology and Evolution* 30 (12): 2725–29. https://doi.org/10.1093/molbev/mst197.

Tashiro, N., S. Uematsu, M. Matsuzaki, Y. Ide, and T. Etoh. 2002. "(124)Phytophthora Palmivora, P. Citrophthora and P. Nicotianae as Causal Agents of Citrus Brown Rot." *Annals of the Phytopathological Society of Japan* 68 (2): 189. http://ci.nii.ac.jp/naid/110002752714/en/.

Tewoldemedhin, Yared Tesfai, Mark Mazzola, Wilhelm J. Botha, Christoffel F.J. Spies, and Adéle McLeod. 2011. "Characterization of Fungi (Fusarium and Rhizoctonia) and Oomycetes (Phytophthora and Pythium) Associated with Apple Orchards in South Africa." *European Journal of Plant Pathology* 130 (2): 215–29. https://doi.org/10.1007/s10658-011-9747-9.

Tsykun, T., C. Rellstab, C. Dutech, G. Sipos, and S. Prospero. 2017. "Comparative Assessment of SSR and SNP Markers for Inferring the Population Genetic Structure of the Common Fungus Armillaria Cepistipes." *Heredity* 119 (5): 371–80. https://doi.org/10.1038/hdy.2017.48.

Vélez, M. L., M. P A Coetzee, M. J. Wingfield, M. Rajchenberg, and A. G. Greslebin. 2014. "Evidence of Low Levels of Genetic Diversity for the Phytophthora Austrocedrae Population in Patagonia, Argentina." *Plant Pathology* 63 (1): 212–20. https://doi.org/10.1111/ppa.12067.

Verhoeven, Koen J.F., Mirka MacEl, Lorne M. Wolfe, and Arjen Biere. 2011. "Population Admixture, Biological Invasions and the Balance between Local Adaptation and Inbreeding Depression." *Proceedings of the Royal Society B:*

Biological Sciences 278 (1702): 2–8. https://doi.org/10.1098/rspb.2010.1272.

Vieira, Maria Lucia Carneiro, Luciane Santini, Augusto Lima Diniz, and Carla de Freitas Munhoz. 2016. "Microsatellite Markers: What They Mean and Why They Are so Useful." *Genetics and Molecular Biology* 39 (3): 312–28. https://doi.org/10.1590/1678-4685-GMB-2016-0027.

Wan, Qiu Hong, Hua Wu, Tsutomu Fujihara, and Sheng Guo Fang. 2004. "Which Genetic Marker for Which Conservation Genetics Issue?" *Electrophoresis* 25 (14): 2165–76. https://doi.org/10.1002/elps.200305922.

Wang, K X, Y L Xie, G Q Yuan, Q Q Li, and W Lin. 2015. "First Report of Root and Collar Rot Caused by Phytopythium Helicoides on Kiwifruit (Actinidia Chinensis)." *Plant Disease* 99 (5): 725. https://doi.org/10.1094/PDIS-08-14-0817-PDN.

Watanabe, Hideki, Yoshihiro Taguchi, Mitsuro Hyakumachi, and Koji Kageyama. 2007. "Pythium and Phytophthora Species Associated with Root and Stem Rots of Kalanchoe." *Journal of General Plant Pathology* 73 (2): 81–88. https://doi.org/10.1007/s10327-006-0338-0.

Whisson, S. C., A. Drenth, D. J. Maclean, and J. A.G. Irwin. 1994. "Evidence for Outcrossing in Phytophthora Sojae and Linkage of a DNA Marker to Two Avirulence Genes." *Current Genetics* 27 (1): 77–82. https://doi.org/10.1007/BF00326582.

Wu, M., B. Li, P. Liu, Q. Weng, J. Zhan, and Q. Chen. 2017. "Genetic Analysis of Phytophthora Sojae Populations in Fujian, China." *Plant Pathology* 66 (7): 1182–90. https://doi.org/10.1111/ppa.12666.

Wu, Xiao ling, Bao qiang Zhang, Shi Sun, Jin ming Zhao, Feng Yang, Na Guo, Jun yi Gai, and Han Xing. 2011. "Identification, Genetic Analysis and Mapping of

Resistance to Phytophthora Sojae of Pm28 in Soybean." *Agricultural Sciences in China* 10 (10): 1506–11. https://doi.org/10.1016/S1671-2927(11)60145-4.

Y., Li, Lee T., Zhu J H., Jin G H., Lan C Z., Zhu S X., Zhang R F., et al. 2012. "Population Structure of Phytophthora Infestans in China – Geographic Clusters and Presence of the EU Genotype Blue_13." *Plant Pathology* 62 (4): 932–42. https://doi.org/10.1111/j.1365-3059.2012.02687.x.

Ye, Jian, George Coulouris, Irena Zaretskaya, Ioana Cutcutache, Steve Rozen, and Thomas L. Madden. 2012. "Primer-BLAST: A Tool to Design Target-Specific Primers for Polymerase Chain Reaction." *BMC Bioinformatics* 13: 134. https://doi.org/10.1186/1471-2105-13-134.

Yin-Ling, Wei Zhou, Keiichi Motohashi, Haruhisa Suga, Hirokazu Fukui, and Koji Kageyama. 2009. "Development of Microsatellite Markers for Pythium Helicoides: RESEARCH LETTER." *FEMS Microbiology Letters* 293 (1): 85–91. https://doi.org/10.1111/j.1574-6968.2009.01518.x.

Yokota, Satoko, Takanori Oomori, Masahiro Nao, Takashi Watanabe, and Hiroko Kitamoto. 2013. "Involvement of Phytophthora Rot Caused by Phytophthora Nicotianae in Growth Failure of Asparagus (Asparagus Officinalis L.) in Replanted Fields in Ehime Prefecture." *Soil Microorganism* 67 (2): 77–82. https://www.jstage.jst.go.jp/article/jssm/67/2/67_KJ00009987127/_pdf/-char/en.

Zane, L., L. Bargelloni, and T. Patarnello. 2002. "Strategies for Microsatellite Isolation: A Review." *Molecular Ecology* 11 (1): 1–16. https://doi.org/10.1046/j.0962-1083.2001.01418.x.