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Mitochondrial DNA polymorphisms in *Phytophthora infestans*: New haplotypes are identified and re-defined by PCR

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ABSTRACT

Polymorphisms of mitochondrial DNA (mt-DNA) are particularly useful for monitoring specific pathogen populations like *Phytophthora infestans*. Basically type I and II of *P. infestans* mt-DNA were categorized by means of polymorphism lengths caused by an ~2 kb insertion, which can be detected via restriction enzyme digestion. In addition genome sequencing of haplotype Ib has been used as a simple Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) method to indirectly identify type I and II alterations through *EcoR* I restriction enzyme DNA fragment patterns of the genomic P4 area. However, with the common method, wrong mt-DNA typing occurs due to an *EcoR* I recognition site mutation in the P4 genomic area. Genome sequencing of the four haplotypes (Ia, Ib, IIa, and IIb) allowed us to thoroughly examine mt-DNA polymorphisms and we indentified two hypervariable regions (HVRs) named HVRi and HVRii. The HVRi length polymorphism caused by a 2 kb insertion/deletion was utilized to identify mt-DNA types I and II, while another length polymorphism in the HVRii region is caused by a variable number of tandem repeats ($n = 1, 2, or 3$) of a 36 bp sized DNA stretch and was further used to determine mt-DNA sub-types, which were described as $R_{n=1,\ 2,\ or\ 3}$. Finally, the *P. infestans* mt-DNA haplotypes were re-defined as IR_1 or IIR_2 according to PCR derived HVRi and HVRii length polymorphisms. Twenty-three isolates were chosen to verify the feasibility of our new approach for identifying mt-DNA haplotypes and a total of five haplotypes (IR_1, IR_2, IR_3, IIR_2 and IIR_3) were identified. Additionally, we found that six isolates determined as type I by our method were mistakenly identified as type II by the PCR–RFLP technique. In conclusion, we propose a simple and rapid PCR method for identification of mt-DNA haplotypes based on sequence analyses of the mitochondrial *P. infestans* genome.

1. Introduction

Potato, the third largest food crop after wheat and rice in the world, suffers from destructive late blight, which results in global yield losses of 16% (Haverkort et al., 2009). Potato late blight is caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, which was responsible for Ireland's potato famine in the 1840s (Avila-Adame et al., 2006). Polymorphisms of mitochondrial DNA in *P. infestans* are particularly useful for epidemiological (Carter et al., 1990), population origin and migration studies (May and Ristino, 2004) as well as for diversity mapping of this pathogen (Gavino and Fry, 2002) because they are uniparentally inherited (Whittaker et al., 1994). Polymorphisms previously have been detected by restriction enzyme digestion patterns of isolated mitochondrial DNA (Carter et al., 1990), PCR–RFLP analysis of mitochondrial DNA (Griffith and Shaw, 1998) and hybridization of digested total DNA with cloned and labeled mt-DNA (Moller et al., 1993). Carter et al. (1990) used RFLP analyses to categorize four mt-DNA haplotypes (Ia, Ib, IIa, and IIb) among 24 isolates from 11 countries (Carter et al., 1990) and at nearly the same time Goodwin (1991) described four haplotypes (A, B, C, and D) derived by Southern blot analyses among 173 isolates from Mexico ($n = 86$), Peru ($n = 15$) and Europe ($n = 36$) (Goodwin, 1991). In addition, Koh et al. found two additional haplotypes (E and F) using Goodwin's method during assaying 124 isolates from East Asian countries (Koh et al., 1994). In a subsequent study, Griffith and Shaw (1998) used sequencing of the mitochondrial haplotype Ib to develop a PCR–RFLP method (Griffith and Shaw, 1998) for rapid identification of the four Carter haplotypes, which has been extensively used to determine mitochondrial DNA haplotypes of *P. infestans* around the world (Chowdappa et al., 2013; Hu et al., 2012; Knapova and Gisi, 2002). Recently the new mitochondrial haplotype Ic has been related to the lineage EC-2 and was assigned into the new species *Phytophthora andina* (Gómez-Alpizar et al., 2007; Ordoñez et al., 2000). Historically, the PCR–RFLP method defined five haplotypes, but on the sequence levels a much greater diversity was observed, because the five mt-DNA loci sequence analyses of 101 isolates derived from

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different areas identified 36 haplotypes, which broadly matched the traditional types I and II but comprised additional subgroups (Martin et al., 2012). Similarly, 176 single nucleotide polymorphisms (SNP) were observed in alignment datasets of the four published mitochondrial genomes (see Supplemental Fig. S1), and at least 176 haplotypes could be determined as sub-populations of *P. infestans*. Restriction enzyme analyses are unsuitable for haplotype classifications due to convergent evolutions at restriction enzyme recognition sites, which do not reflect evolutionary divergence across the mitochondrial genomes (Martin et al., 2012). The hypervariable regions (HVRs) caused by insertion/deletion and more specifically designated as HVRi and HVRii have been located in the haplotype Ib mitochondrial genome (Fig. 1) during alignment analyses of genome sequences. Of these, HVRi includes the 2 kb insertion (1885 bp in size) and HVRii comprises a variable tandem repeat number (36 bp in size (n = 1, 2, or 3 = repeat number)). Length polymorphisms of PCR amplicons from HVRi and HVRii have been used to re-define the mt-DNA haplotypes of *P. infestans*. The aim of this research was to further simplify the identification of *P. infestans* mt-DNA type I and II and propose an improved general method for rapidly identifying mt-DNA haplotypes by PCR.

2. Materials and methods

2.1. Isolates

Single-zoospore isolates were maintained on rye A 1.5% agar slants at 10 °C and were transferred every 6 months. Their codes, origin, year and sample number in the following experiments are listed in Table 1. Colony diameters were observed every 8–10 d for transferring one 0.5-cm colony from a slant cube to a rye A petri plate and incubation at 18 °C. All plate cultures (appropriate 5-cm diameter colonies) could provide sufficient amounts of mycelia and sporangia for DNA extraction.

2.2. DNA extraction and PCR procedure

Total genomic DNA of *P. infestans* isolates was extracted, using a modified Cetyltrimethylammonium bromide (CTAB) method (Trout et al., 1997). DNA was diluted 1:10 (5–10 ng/μl) for PCR amplifications, which were carried out in 25 μl reaction volume containing 12.5 μl 2× Es Taq Mastermix (CoWin Biotech Co. Ltd., Beijing, China), 9.5 μl sterile distilled H_2O, 1 μl of each primer, and 1 μl DNA template. The thermal cycling conditions for HVRi consisted of an initial denaturation at 95 °C for 2 min, and 27 cycles of 95 °C for 45 s, 64 °C for 3 min, and 72 °C for 3 min with a final extension at 72 °C for 10 min. The thermal cycling conditions for HVRii consisted of an initial denaturation at 95 °C for 2 min, and 30 cycles of 95 °C for 45 s, 45 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 7 min. The HVRi and HVRii PCR products were separated by electrophoresis in 1.0% and 2.0% (wt/vol) TBE buffer agarose gels and subsequently visualized by UV illumination after ethidium bromide staining. DNA markers MD5000 or MD500 (CoWin Biotech Co. Ltd., Beijing, China) were included on the gels as molecular size standards.

Table 1
Single-lesion isolates of *P. infestans* used in this study.

Isolate code	Collection site	Year	Serial no.[a]
MJ08-07	Fujian, China	2008	1
MJ08-11	Fujian, China	2008	2
SXt99-01	Shanxi, China	1999	3
HH00-04	Heilongjiang, China	2007	4
HM09-01	Heilongjiang, China	2009	5
HK06-28	Heilongjiang, China	2006	6
JM09-06	Heilongjiang, China	2006	7
HK06-51	Heilongjiang, China	2006	8
HK09-01	Jilin, China	2009	9
YK09-23	Yunnan, China	2009	10
CQ08-03	Chongqing, China	2008	11
NG10-15	Ningxia, China	2010	12
DK98-02	Yunnan, China	1998	13
SW96-01	Switzerland	2008	14
US05-01	United State of America	2008	15
US05-08	United State of America	2008	16
NG10-84	Ningxia, China	2010	17
JW04-15	Hebei, China	2004	18
JW04-08	Hebei, China	2004	19
JW07-03	Hebei, China	2007	20
JW04-10	Hebei, China	2004	21
JD09-37	Jilin, China	2009	22
JW11-08	Inner Mongolia, China	2011	23

[a] Serial number of samples in the following experiments.

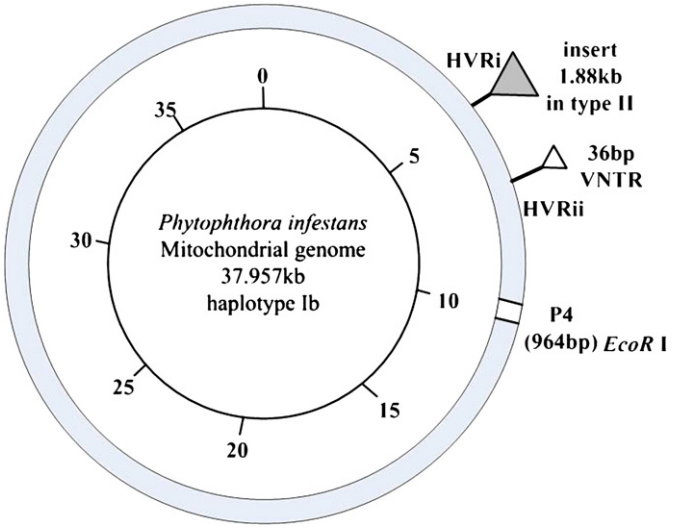

Fig. 1. Diagram representation of the mitochondrial *P. infestans* genome illustrating the location of HVRi and HVRii for redefining mt-DNA haplotypes. The position of a ca. 1.88 kb insert present in haplotype II is indicated as HVRi. The location of 36 bp VNTRs in the genome is indicated as HVRii. P4 is the F4–R4 PCR product (Griffith and Shaw (1998)).

2.3. Mitochondrial DNA type I and II detection by PCR–RFLP

DNA of each isolate was amplified using specific primers (F4–R4) designed for the P4 region of the mitochondrial *P. infestans* genome. Digestion of P4 products with the restriction enzyme *EcoR*I yielded band patterns by which the isolates could be classified into the two different types: I(a/b) and II(a/b) (Griffith and Shaw, 1998).

3. Results

3.1. Length polymorphisms of HVRi and HVRii mt-DNA for haplotype classifications

The positions of HVRi and HVRii in the mitochondrial genome of *P. infestans* are shown in Fig. 1. The HVRi including a 1885 bp insertion was chosen to directly identify the traditional types I and II by PCR. The HVRii contains also a variable number of tandem repeats (VNTR) (n = 1, 2, or 3) of a 36 bp insert (5′-GAAAAAAAAATTATAA CAAGT TAATTTAAAATAGAT-3′) and thus three new mt-DNA types (type R_n; n = 1, 2, or 3) were also determined by VNTR analyses. The variable number of tandem repeats (R_n) is directly correlating with the length of the amplicons. Finally, additional new mt-DNA sub-haplotypes of *P. infestans* were defined as IR_n and IIR_n (n = 1, 2, or 3).