

NOTE

Mitochondrial Phylogeny Reveals Intraspecific Variation in *Peronospora effusa*, the Spinach Downy Mildew Pathogen

Young-Joon Choi¹, Marco Thines^{2,3}, Jae-Gu Han⁴, and Hyeon-Dong Shin^{4*}

¹Harvard University, Department of Organismic and Evolutionary Biology, 22 Divinity Ave., Cambridge, MA 02138, USA

²Johann Wolfgang Goethe University, Department of Biological Sciences, Institute of Ecology, Evolution and Diversity, Siesmayerstr 70, D-60323 Frankfurt (Main), Germany

³Biodiversity and Climate Research Centre (BiK-F), Senckenberganlage 25, D-60325 Frankfurt (Main), Germany

⁴Korea University, Division of Environmental Science and Ecological Engineering, Seoul 136-701, Republic of Korea

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Since about two hundred years, downy mildew caused by *Peronospora effusa* is probably the most economically important disease of spinach (*Spinacia oleracea*). However, there is no information on the global phylogeographic structure of the pathogen and thus it is unclear whether a single genotype occurs worldwide or whether some local genetic variation exists. To investigate the genetic variability of this pathogen, a sequence analysis of two partial mitochondrial DNA genes, *cox2* and *nad1*, was carried out. Thirty-three specimens of *Peronospora effusa* from four continents were analyzed, including samples from Australia, China, Japan, Korea, Mexico, Russia, Sweden, and the USA. Despite the potential anthropogenic admixture of genotypes, a phylogeographic pattern was observed, which corresponds to two major groups, an Asian/Oceanian clade and another group, which includes American/European specimens. Notably, two of six Japanese specimens investigated did not belong to the Asian/Oceanian clade, but were identical to three of the specimens from the USA, suggestive of a recent introduction from the USA to Japan. As similar introduction events may be occurring as a result of the globalised trade with plant and seed material, a better knowledge of the phylogeographic distribution of pathogens is highly warranted for food security purposes.

Keywords: obligate parasites, Oomycetes, phylogeographic distribution, plant pathogen, quarantine

The family Peronosporaceae consists of the hemibiotrophic *Phytophthora* and obligate biotrophic parasitic downy mildew pathogens (Thines, 2009) that cause disease on a large number of cultivated and wild angiosperm hosts. In the cultivation of spinach (*Spinacia oleracea*), an increasingly popular leafy vegetable crop, it is probably the most devastating disease and has been reported in most countries where the crop is cultivated (Byford, 1981; Correll *et al.*, 1994). After the first record of the spinach downy mildew by Greville (1824), disease occurrences have been continuing up to now. However, both the host range of the pathogen and the question of whether a single pathogen causes the disease on spinach worldwide have been controversially discussed, possibly due to complex taxonomy and nomenclature of this oomycete (Brandenberger *et al.*, 1991). Recently, it was proven that the spinach downy mildew pathogen is a highly specialized species that is delimited from pathogens of other chenopodiaceous hosts based on both morphological and molecular phylogenetic evidence and accordingly the name *Peronospora effusa* (Grev.) Rabenh. had been reinstated (Choi *et al.*, 2007a). However, due to the homogeneity of the specimens in ITS sequences, information on the genetic diversity of the pathogen is so far lacking.

Such data is important to both seed companies and spinach producers, who effort to develop disease-resistant cultivars, which are the most common management practice for the disease. Besides, information on the global phylogeographic structure is crucial for regulatory authorities which aim at preventing the inadvertent intercontinental introduction of genetically unique foreign populations as a result of global trade activities (Brasier *et al.*, 1999; O'Donnell *et al.*, 2000)

From initial investigation of ITS rDNA region, all *Peronospora effusa* specimens showed no sequence variation (Choi *et al.*, 2007a). Although the ITS sequences have been most commonly used in taxonomy of the downy mildews (reviewed in Voglmayr, 2008), the lack of ITS variation does not always automatically confirm the genetic homogeneity. Only by using other genes exhibiting more resolution, including variable protein-coding genes, a clear delimitation of these species is possible. In light of previous results that demonstrated the usefulness of the multigene sequence analysis of downy mildews (Göker *et al.*, 2007) and that showed the high efficiency of PCR amplification and sequencing for mitochondrial DNA of historic specimens (Telle and Thines, 2008), we conducted phylogenetic sequence analysis of *cox2* and *nad1* mtDNA genes of both recent and historic specimens. The present study aimed at investigating whether the *P. effusa* is equally uniform in regions more variable than the previously studied

* For correspondence. E-mail: hdshin@korea.ac.kr; Tel.: +82- 2-3290-3063; Fax: +82-2-921-1715

internal transcribed spacers or whether the spinach downy mildew pathogens from a worldwide collection would exhibit some phylogeographic structure important for quarantine and risk assessment.

Molecular sequence analysis was carried out with 33 *Peronospora effusa* specimens collected or loaned from herbaria BPI, DM, HMAS, KUS-F, LE, RD, UPS, and VPRI. The specimens originated from eight countries of Asia (China, Japan, Korea), Europe (Sweden, Russia), North America (USA, Mexico), and Oceania (Australia). Information on specimens sequenced in this study is given in Table 1. Herbarium abbreviations are those from Thiers (2011).

Genomic DNA was extracted from conidiophores and conidia on the lower surface of the infected leaves of herbarium specimens. DNA extraction was performed using the methodology described in Lee and Taylor (1990). Amplification of the partial *cox2* and *nad1* mtDNA regions was carried out in a PTC-200 thermal cycler (MJ Research, USA) using a high-fidelity *Taq* polymerase, EX-*Taq* (TaKaRa, Japan). Primer sets and conditions designed by Hudspeth *et al.* (2000) and Kroon *et al.* (2004) were employed, respectively. The sequencing

runs of the double strands were performed using an ABI 3730XL DNA sequencer (AB, USA) and were analyzed by ABI Sequencing Analysis software v 5.1 and KB Basecaller (both from AB) capable of sequence quality value (QV) prediction on each base call by a commercial sequencing company (Macrogene, Korea), with the primers used for amplification. The robustness of PCR and sequencing results for single base-pair substitution was confirmed by using a different thermal cycler (Swift Maxi Thermal Cycler, ESCO Technologies, USA) and sequencing company (SolGent, Korea). For a USA specimen (BPI791055) that showed overlapping peaks at a position of *nad1* mtDNA sequences, the purified PCR products were ligated into pGEM-T Easy Vector (Promega, USA) and transformed into *Escherichia coli* DH5 α F' cells. Twenty clones were randomly selected and plasmids containing the *nad1* regions were isolated using a QIAquick Plasmid Minikit (Promega), and pooled for diminishing errors introduced by the *Taq* DNA polymerase used for PCR reactions. Sequences were edited with the DNASTAR computer package (DNASTar, Inc., USA), version 5.05. Alignment using the ClustalW algorithm was performed and viewed in MEGA 4.0 (Tamura *et al.*, 2007). Phylogenetic trees were obtained from the concatenated alignment of the two mitochondrial regions using Minimum Evolution (ME) and Maximum Parsimony (MP) methods as implemented in MEGA 4.0 with default parameters, for which 1000 bootstrapping (BS) replicates were performed.

The alignment lengths for *cox2* and *nad1* segments were 509 bp and 519 bp, respectively. No indels were found in both alignments. An intraspecific sequence variation of three base substitutions was observed in each mitochondrial gene, all of which were transitions (Fig. 1). The phylogenetic analysis of the concatenated alignments consisting of *cox2* and *nad1* sequences was inferred using ME and MP methods. Since no differences were found between the resulting tree topologies, only the former tree is shown in Fig. 2. Using a complete mitochondrial sequence of *Phytophthora sojae* (DQ832717) as a reference for a careful base-by-base comparison, the substitutions among the sequences were mapped on the branches of ME tree.

In the *cox2* mtDNA region, the sequences were divided into two groups; Group I for Asian and Oceania specimens with ME BS (90%) and MP BS (92%) and Group II for American and European ones with the exception of two Japan specimens (DM65 and DM69) with ME BS (78%) and MP BS (88%). In Group I, the Asian specimens, from the China (4), Japan (5), and Korea (5), formed a group with Australian ones (6). All Australian specimens further formed a subgroup, excluding one clustered within the Asian specimens. The Korean specimens were divided into two subgroups, of which three were identical to other Asian specimens, while two formed a distinct branch. Within Group I, two substitutions were found, a change from C to T at position 468 for Australian specimens and from A to G at 528 for the Korean ones. All American/European specimens of Group II, which originated from Mexico (2), Russia (1), Sweden (2), and the USA (5), were identical, and differed at position 570 from Asian/Oceania group. Similarly, the *nad1* sequences allowed the separation between Group I and II, with a transition at position 535. All members of Group I were identical in *nad1* sequences. The American/European specimens were divided

Table 1. *Peronospora effusa* specimens used in this study

Geographical origin/year of collection (source or herbarium number)	GenBank acc. no. COX2 / ND1
Australia, Leppington, 2002 (VPRI30202)	FJ649395 / FJ649443
Australia, V.I.C., 1991 (VPRI17377)	FJ649396 / FJ649444
Australia, V.I.C., 1999 (VPRI21915)	FJ649397 / FJ649445
Australia, V.I.C., 2000 (VPRI22523)	FJ649398 / FJ649446
Australia, V.I.C., 2003 (VPRI30651)	FJ649399 / FJ649447
Australia, V.I.C., 2003 (VPRI31625)	FJ649400 / FJ649448
China, Jiangsu, 1980 (HMAS57061)	FJ649401 / FJ649449
China, Sichuan, 1958 (HMAS57074)	FJ649402 / FJ649450
China, Yunnan, 1974 (HMAS57079)	FJ649403 / FJ649451
China, Yunnan, 1983 (HMAS57076)	FJ649404 / FJ649452
Japan, Fukui, 2000 (DM68)	FJ649405 / FJ649453
Japan, Gifu, unknown (DM22)	GU292561 / GU292562
Japan, Hokkaido, 2004 (DM81)	FJ649406 / FJ649454
Japan, Kanagawa, 2002 (DM78)	FJ649407 / FJ649455
Japan, Shiga, 2000 (DM65)	FJ649408 / FJ649456
Japan, Shiga, unknown (DM34)	FJ649409 / FJ649457
Japan, Shimane, 2000 (DM69)	FJ649410 / FJ649458
Korea, Busan, 2007 (KUS-F23198)	FJ649411 / FJ649459
Korea, Namyangju, 1998 (KUS-F15610)	FJ649412 / FJ649460
Korea, Namyangju, 1999 (KUS-F15680)	FJ649413 / FJ649461
Korea, Sinan, 2001 (KUS-F18808)	FJ649414 / FJ649462
Korea, Sinan, 2001 (KUS-F18809)	FJ649415 / FJ649463
Mexico, Nogales, 1949 (BPI788314)	FJ649416 / FJ649464
Mexico, Nogales, 1953 (BPI788308)	FJ649417 / FJ649465
Russia, Kursk, 1882 (LE187818)	FJ649418 / FJ649466
Sweden, Gästrikland, 1942 (UPS)	FJ649419 / FJ649467
Sweden, Stockholm, 1962 (LE187806)	FJ649420 / FJ649468
USA, Kingston, 1896 (UPS)	FJ649421 / FJ649469
USA, Maryland, 1915 (BPI788312)	FJ649422 / -
USA, Maryland, 1951 (BPI788309)	FJ649423 / FJ649470
USA, Maryland, 1958 (BPI788300)	FJ649424 / FJ649471
USA, Oklahoma, 1943 (BPI791055)	FJ649425 / FJ649472
USA, Virginia, 1932 (BPI788361)	FJ649426 / FJ649473

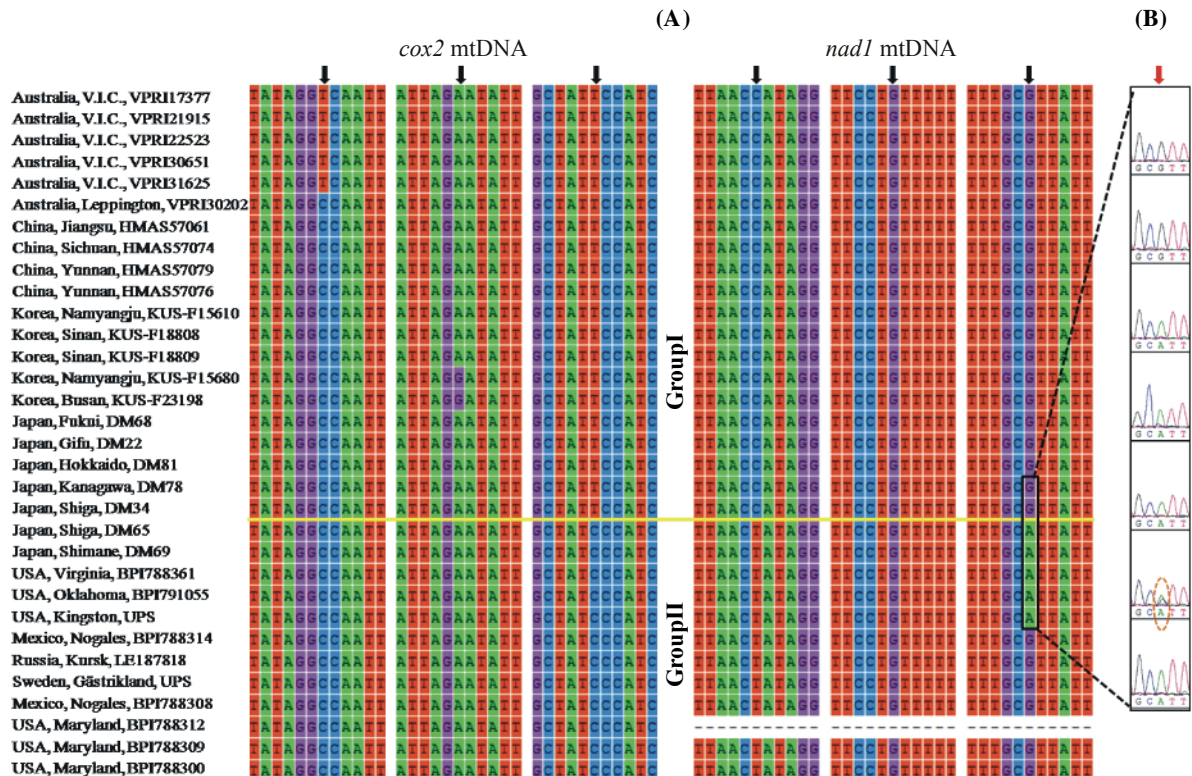


Fig. 1. Sequence alignment of the partial *cox2* and *nad1* mtDNA sequences of *Peronospora effusa*. (A) Location of substitutions within *cox2* and *nad1* mtDNA regions. Arrow indicates the site of substitution. (B) Chromatograms of *nad1* sequences of Japan and USA specimens clustered within a group. Note a USA specimen (BPI791055) with overlapped peaks in red circle.

into two subgroups based on *cox2* sequences. Two substitutions allowed the subdivision into three genotypes, a change from G to A at two positions 307 and 429, respectively; the former change divided the USA samples into two subgroups, of which one clustered with two Japanese ones, while the latter change distinguished a Sweden sample from other European samples. One specimen from the USA (BPI791055) showed an overlapping peak of the bases A and G in the *nad1* gene (Fig. 1B), a cloning of the PCR products was performed to distinguish the bases present in single, cloned copies of the gene array. It was revealed that the base is dimorphic site; among 20 clones, 12 clones had an A at position 429, while the others had a G at the corresponding position, indicating that it had mitochondria of both genotypes and therefore represents an intermediate between the American/ European and the Japanese/USA subgroups.

In the present analysis of two partial mitochondrial genes, *cox2* and *nad1*, we could demonstrate that the spinach downy mildew pathogen, *Peronospora effusa*, is not consisting of a single genotype of worldwide distribution, but contains several haplotypes for both genes investigated. This could either be the result of recent divergence after anthropogenic spread or be due to the colonisation of cultivated spinach from different genotypes, which already resided in the Northern hemisphere on species closely related to *Spinacia oleracea*. Interestingly, the cloning analysis of the *nad1* mtDNA indicated that an intraspecific hybrid is present between two genotypes, the

Japanese/USA and American/European genotype, which harbours both mitochondrial genotypes at a very similar frequency. The fact that *P. effusa* populations with different genotypes are causing downy mildew disease on spinach, even in the same country, has some implications for spinach production. At present, this disease is managed mainly by planting resistant cultivars, which are continuously bred for higher resistance, which could potentially be overcome by spontaneous mutations in the pathogen or the introduction of new pathogen genotypes. Therefore, the finding that various genotypes are already distinguishable on the basis of standard markers used in molecular phylogenies should be considered by both breeding companies and spinach producers for downy mildew control strategies. In particular, resistance of new spinach cultivars should ideally be tested against all genotypes revealed in this study, for ensuring a sustainable resistance. A phylogeographic pattern of the haplotypes was revealed with all American/ European spinach downy mildew specimens were placed in Group II, while, with the exception of two Japanese specimens, the Asian/Oceanian specimens also clustered together in Group I. The placement of two of the Japanese specimens into the group otherwise only containing samples from the western hemisphere might have occurred recently by an anthropogenic introduction from the USA to Japan. This hypothesis is in line with the recent reports that new physiological races of *P. effusa* are endangering spinach cultivation in Japan (Satou *et al.*, 2002, 2006), which would be interesting to follow

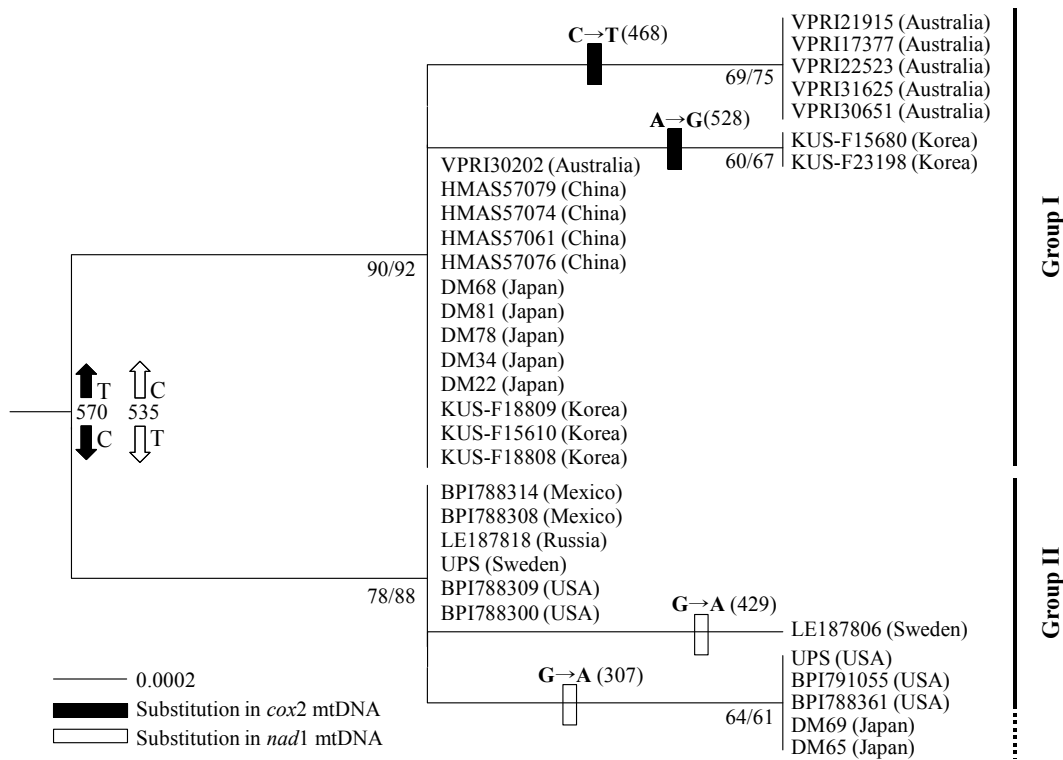


Fig. 2. Diagnostic substitutions mapped on the ME tree topology of concatenated alignment of *cox2* and *nad1* mtDNA. The positions of substitutions in the two genes are numbered in parentheses. The branch lengths are proportional to the characters that change unambiguously on the branches. Supporting values (ME BS/MP BS) above 50 % are given below the branches.

up with a more detailed sampling. Similar patterns can be expected in other species, and Choi *et al.* (2009a) suggested that ragweed downy mildew (*Plasmopara angustiterminalis*) pathogen in Europe (Hungary) might have migrated from North America, possibly from Canadian populations, based on the sequence analyses of the *cox2* and *nad1* mtDNA genes. These results underline the discriminative power of mitochondrial DNA sequences, which seems to be higher than the ITS region in downy mildews, as no sequence variation in *P. effusa* could be observed on the basis of ITS sequences. Unlike in *Plasmopara* and *Bremia* species, where the ITS2 region is highly variable due to multiple repeat sequences (Thines *et al.*, 2005; Choi *et al.*, 2007b; Thines 2007), the ITS2 region is mostly not suitable for distinguishing genotypes below the level of species in the largest genus of the downy mildews, *Peronospora*. However, mitochondrial loci might serve as an equally variable alternative in this genus.

The fact that substitutions in both mitochondrial genes investigated all are transitions could be an indication that the genetic divergence of *P. effusa* population occurred recently. Spinach has only recently been cultivated in most parts of the world, and high host specificity indicates that the downy mildew pathogen would have been spread together with the crop, starting from a rather small geographic area. This comparatively short period of area expansion, together with high host specificity, may be a likely explanation for the homogeneity of the ITS region previously (Choi *et al.*, 2007a) and the

low sequence variation of two mitochondrial genes presently. To date, many fungal and oomycete pathogens have been reported on spinach (Correll *et al.*, 1994; Farr and Rossman, 2009), among which white blister rust caused by *Albugo occidentalis* (Wiant *et al.*, 1939; Raabe and Pound, 1952) and damping-off caused by *Fusarium oxysporum* (Naiki and Kanoh, 1977; Larsson and Gerhardson, 1992) are diseases of similar impact as downy mildew. Spinach seeds carrying vital spores or mycelium of any of the three pathogens contribute as an important source of primary inoculum and spread of the diseases they cause (Raabe and Pound, 1952; Bassi and Goode, 1978; Inaba and Morinaka, 1984). With the increased worldwide seed trade with several high performance cultivars, the danger of introducing new strains of pathogens is increasing, posing a potential threat to spinach production. At first stages of infection there are usually no clear symptoms developing, especially in infections with *A. occidentalis* and *P. effusa*. In addition, very small amounts of inoculums might already give rise to epidemics with previously unknown pathogens, as was previously observed with basil downy mildew caused by *P. belbahrii* (Thines *et al.*, 2009) or sage downy mildews (Choi *et al.*, 2009b). Therefore, it is unlikely that these be easily detected by the conventional, mainly visual diagnostic methods still often used in international quarantine. However, it is critically important in the context of global trade with seeds to establish sensitive tests with high discriminative power for addressing the threat posed by the introduction of new species

and strains of crop pathogens. In the present study, two mitochondrial genes, *cox2* and *nad1*, have been sequenced, and diagnostic base substitutions were found that separate *P. effusa* samples into several genotypes. These loci could probably serve to discriminate between existing and to identify new pathogen genotypes in quarantine investigation or field studies.

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