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Re-consideration of *Peronospora farinosa* infecting *Spinacia oleracea* as distinct species, *Peronospora effusa*

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ABSTRACT

Downy mildew is probably the most widespread and potentially destructive global disease of spinach (*Spinacia oleracea*). The causal agent of downy mildew disease on various plants of *Chenopodiaceae*, including spinach, is regarded as a single species, *Peronospora farinosa*. In the present study, the ITS rDNA sequence and morphological data demonstrated that *P. farinosa* from *S. oleracea* is distinct from downy mildew of other chenopodiaceous hosts. Fifty-eight spinach specimens were collected or loaned from 17 countries of Asia, Europe, Oceania, North and South America, which all formed a distinct monophyletic group. No intercontinental genetic variation of the ITS rDNA within *Peronospora* accessions causing spinach downy mildew disease was found. Phylogenetic trees supported recognition of *Peronospora* from spinach as a separate species. Microscopic examination also revealed morphological differences between *Peronospora* specimens from *Spinacia* and *P. farinosa s. lat.* specimens from *Atriplex*, *Bassia*, *Beta*, and *Chenopodium*. Consequently, the name *Peronospora effusa* should be reinstated for the downy mildew fungus found on spinach. Here, a specimen of the original collections of *Peronospora effusa* is designated as lectotype.

Introduction

Peronospora farinosa is an obligate parasite, which causes downy mildew disease on many chenopodiaceous host plants, *viz. Atriplex*, *Beta*, *Chenopodium*, and *Spinacia*. The occurrence of this severe disease on economically important hosts, such as spinach and beet, has been reported in almost all countries where these crops are cultivated since the nineteenth century (Byford 1981). Despite its economic and taxonomic importance, no molecular evidence supporting the homogeneity of *P. farinosa* materials from different host genera and/or species has been provided, because to obtain both good specimens and sequences of those specimens is difficult for obligate parasitic fungi.

Since a downy mildew fungus infecting the family *Chenopodiaceae* was first described by Greville (1824), who referred to the pathogen of *Spinacia* as *Botrytis effusa*, Rabenhorst (1854) transferred *B. farinosa* and *B. effusa* to *Peronospora*. Within *Peronospora* from *Chenopodiaceae*, Caspary (1855) recognized two taxa, var. *major* and var. *minor*. Wilson (1914) recognized the variety 'major' as *Peronospora effusa*, with spinach as the major host and the variety 'minor' as *P. farinosa*, with *Atriplex*, *Chenopodium*, and *Spinacia* as hosts, indicating the difficulty of distinguishing both species using their hosts. Gäumann (1919) found that *Peronospora* species cannot be separated into only two species, and so established 14 different species. In contrast, Yerkes & Shaw (1959) were unable to find any

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suitable morphological differences for division into separate species, and proposed the inclusion of all *Peronospora* species parasitic on *Chenopodiaceae* into a single species, *P. farinosa*. Although these authors abandoned the name *P. effusa* for the spinach downy mildew fungus, they provided no convincing evidence for conspecificity with *P. farinosa*. A good account of the taxonomy and nomenclature of this fungus has been given by Brandenberger *et al.* (1991).

Previous studies have shown that *Peronospora* accessions from *Chenopodiaceae* are host specific (Hiura 1929; Cook 1936; Richards 1939; Darpoux & Durgéat 1962). As a result of cross-inoculation experiments, Byford (1967) subdivided *P. farinosa* into three special forms: f. sp. *betae* on *Beta* spp., f. sp. *chenopodii* on *Chenopodium* spp., and f. sp. *spinaciae* on *Spinacia oleracea*. Despite the clear physiological specialization to their hosts, the uncertain species delimitation among the downy mildew fungi on *Chenopodiaceae* has resulted in serious problems with respect to the correct identification of this pathogen, which should be clarified.

Our previous work (Choi *et al.* 2002) suggested that *Peronospora* accessions originating from *Spinacia* and *Chenopodium* in Korea are morphologically and molecularly different from each other. However, the study included a relatively small number of specimens, which were also geographically limited to Korea. Thus, a further comprehensive study was needed to clarify the systematics and nomenclature of the downy mildew fungus infecting spinach worldwide. In the present study, 58 infected spinach specimens, including 19 from North and South America (Argentina, Canada, Mexico, and the USA), 19 from Asia (China, Iran, Japan, and Korea), 11 from Europe (Austria, Czech Republic, Latvia, Romania, Russia, and Sweden), and nine from Oceania (Australia and New Zealand), were used in a phylogenetic analysis of the ITS region of rDNA. Sequence analysis of the ITS rDNA is a powerful tool for the comparison of closely related species, and has recently been applied to resolve taxonomic and phylogenetic relationships of closely related species within *Peronospora* and allied genera (Constantinescu & Fatehi 2002; Choi *et al.* 2003, 2005; Voglmayr 2003; Göker *et al.* 2004).

Therefore, the main aims of this study were to solve the taxonomic problems of the spinach downy mildew fungus following its separation from *P. farinosa*, and to investigate genetic intercontinental differences within *P. effusa*, based on phylogenetic analyses of the ITS rDNA sequences as well as morphology.

Materials and methods

Fungal specimens

Sequence analysis of ITS rDNA was carried out with 72 *Peronospora* specimens from chenopodiaceous hosts. Of these, 68 dried herbarium specimens were sequenced in the present study, with five further sequences obtained from GenBank. For comparison, 17 additional sequences of *Peronospora* and *Hyaloperonospora* were also included as representatives of the family *Peronosporaceae*. Vouchers of the recently collected specimens were deposited at SMK (Herbarium of Systematic Mycology of Korea, Korea University, Korea). Information on the 89 sequences included in the phylogenetic analysis is listed in Table 1. Herbarium abbreviations are according to Holmgren & Holmgren (http://sciweb.nybg.org/science2/indexHerbariorum.asp).

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted using the conidiophores and conidia formed on the lower or upper surface of the infected leaves, or the infected host tissue of herbarium specimens. The extraction of genomic DNA was performed according to the method of Lee & Taylor (1990). The DC6 (Cooke *et al.* 2000) and ITS4 (White *et al.* 1990) primers were used for selective amplification of the complete ITS region of the rDNA. The success of the amplification was monitored by electrophoresis on 1 % agarose gels, and purified using the QIAquick gel extraction kit (QUIAGEN, Hilden, Germany). Purified DNA was directly sequenced on an automatic sequencer (ABI Prism TM 377 DNA Sequencer), using primers ITS1, ITS2, ITS3, and ITS4 (White *et al.* 1990).

Sequence alignment and phylogenetic analyses

Sequences were edited using the DNAStar (DNAStar, Madison, WI) computer package. An alignment of the sequences was performed using the CLUSTAL W (Thompson *et al.* 1994) program. The 89 sequences were adjusted to the length of the complete ITS region (ITS1, 5.8S rDNA, and ITS2). Phylogenetic trees were obtained from the data using distance, parsimony, and Bayesian methods. For the distance analysis, the most appropriate evolutionary model was determined for a given data set using Modeltest 3.06 (Posada & Crandall 1998) and PAUP version 4b10 (Swofford 2002). Once the general time reversible model (GTR), with gamma-distributed substitution rates, had been chosen, this was used to construct phylogenetic trees employing the NJ method using PAUP. An MP heuristic search was performed with ten random sequence additions, branch swapping by tree bisection–reconnection (TBR), and MAXTREES set at 20K, also using PAUP. Gaps were treated as missing data, with all nucleotide substitutions equally weighted and unordered. The CI and RI were calculated for all MP trees (Kluge & Farris 1969; Farris 1989). The relative robustness of the individual branches was estimated by bootstrapping (BS), using 1K replicates, in both the distance and parsimony analyses. A Bayesian analysis was performed using MRBAYES, version 3.0b4 (Ronquist & Huelsenbeck 2003). This program performs a Bayesian inference of the phylogeny, using Metropolis-coupled MCMC (MC3; Geyer 1991) analyses. Four incrementally heated simultaneous Markov chains were run for 1M generations, with a tree saved every 100th generation. The first 1K trees generated via this method were ignored. MRBAYES was used to compute a 50 % majority rule consensus of the remaining trees to obtain estimates for the PPs of groups. Branch lengths were computed as the mean values over the trees sampled. To test the reproducibility of the results, this analysis was repeated four times, starting with random trees and default parameter values. Trees were rooted using the TREEVIEW program, version 1.6.6 (Page 1996), by selecting *Hyaloperonospora parasitica* as outgroup.