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Variation in the nrDNA ITS sequences of some powdery mildew species: do routine molecular identification procedures hide valuable information?

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Abstract During the past years, nrDNA ITS sequences have supported the identification of many powdery mildew fungi because comprehensive analyses showed that differences in these sequences have always correlated with the delimitation of different species and formae speciales of the *Erysiphales*. Published data, obtained using direct sequencing of the PCR products, suggested that even one to five nucleotide differences in the ITS sequences delimit different, albeit closely related, species, and/or indicate differences in host range patterns. Here we show that such differences in the ITS sequences can be detected even in a single sample of a powdery mildew fungus. We sequenced the ITS region in 17 samples, representing six powdery mildew species, both directly and after cloning the PCR products. Among these, samples of *O. longipes* exhibited two or three, samples of *O. neolycopersici* three or four, those of an *Oidium* sp. from *Chelidonium majus* up to seven, and a sample of another *Oidium* sp. from *Passiflora caerulea* two different ITS types determined after cloning. No ITS nucleotide polymorphisms were found in samples of *O. lycopersici* and *Erysiphe aquilegiae*. This suggests that some powdery mildew taxa are more variable at the ITS level than others. Thus, although the ITS sequences determined by direct sequencing represent robust data useful in delimitation and phylogenetic analysis of distinct species of the *Erysiphales*, these need to be used with precaution, and preferably determined after cloning, especially when dealing with closely related taxa at species and sub-species levels. With this method a hitherto undetected genetic diversity of powdery mildews can be revealed.

Keywords Erysiphales · GenBank · ITS polymorphism · paralogue sequences

Introduction

Powdery mildew fungi (*Ascomycota: Erysiphales*) are widespread pathogens of many plant species, including important crops such as wheat, barley, grapevine, apple, various vegetables, ornamentals, and so on. The identification of powdery mildew species is based on morphological characteristics of the anamorph and teleomorph stages and also on the identity of the host plant species. In addition, during the past years, the internal transcribed spacer (ITS) sequences of the

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nuclear ribosomal DNA (nrDNA) have also helped the identification of many species because all the studies have shown that differences in the nrDNA ITS sequences always correlated with the delimitation of different species and formae speciales of the *Erysiphales*. These sequences were always identical or 98–99% similar within, but always differed among, all the well-defined powdery mildew species and formae speciales identified based on morphological and/or host range criteria (e.g., Cook et al. 2006; Inuma et al. 2007; Kiss et al. 2008; Shiroya and Takamatsu 2009; Takamatsu et al. 2009; Heluta et al. 2009). Phylogenetic relationships among various powdery mildew species have also been elucidated mainly based on ITS, and 28S and 18S, nrDNA sequences (e.g., Matsuda and Takamatsu 2003; Cunnington et al. 2005; Kiss et al. 2006; Takamatsu et al. 2008, 2009; Ito and Takamatsu 2010). Comprehensive phylogenetic analyses based on DNA sequences other than nrDNA have only been carried out in the genus *Blumeria* infecting monocots (Inuma et al. 2007) and in the grapevine powdery mildew pathogen, *Erysiphe necator* (Brewer and Milgroom 2010). However, these multi-locus analyses did not reveal any additional markers that could be used as universally as the ITS region in the molecular identification of powdery mildew taxa at species and sub-species levels.

Based on these findings, it has become a common practice to determine the ITS sequences in newly described powdery species (e.g., Shiroya and Takamatsu 2009; Takamatsu et al. 2009; Beilharz et al. 2010) and also to identify newly found powdery mildew pathogens based on their ITS sequences especially when morphological and/or host range data were scarce, and, thus, insufficient for precise identification purposes (e.g., Cunnington et al. 2003; Kiss et al. 2005; Jankovics et al. 2008). This latter situation has arisen quite frequently during the past years because some powdery mildew fungi have recently become invasive in certain areas (Cook et al. 2006; Seko et al. 2008; Heluta et al. 2009), and/or have shown up on plant species which have not been previously reported to host them (e.g., Vági et al. 2007; Jankovics et al. 2008). In such cases, a portion of the species do not produce the teleomorph stage regularly, or do not produce them at all, and this makes their identification difficult (Cunnington et al. 2003; Takamatsu et al. 2007; Vági et al. 2007; Jankovics et al. 2008; Kiss et al. 2008; Seko et al. 2008).

In all the above mentioned studies, the ITS sequences were determined using direct sequencing of the PCR products. However, it is well-known that sequencing errors might occur during this process (Bridge et al. 2003; Simon and Weiss 2008) and this could be complicated by the presence of multiple copies of the ITS region in the nuclear genomes. Every nuclear genome contains a high number of ITS sequences and in some ascomycetous fungi, similar to other organisms, the presence of divergent intragenomic ITS sequences has already been demonstrated (e.g., O'Donnell and Cigelnik 1997; Nilsson et al. 2008; Simon and Weiss 2008; Kovács et al. 2011). In powdery mildew fungi, such variation in the ITS sequences is not known.

Recently, a comprehensive study has shown that one to five nucleotide differences in the ITS sequences of morphologically indistinguishable powdery mildew anamorphs reflect considerable differences in their genome-wide diversity, assessed using AFLP analysis, and also in their host ranges, determined in cross-inoculation tests (Jankovics et al. 2008). In fact, some of the morphologically similar mitosporic powdery mildew species do not differ in their ITS sequences in more than one to a few nucleotides (e.g., Kiss et al. 2005, 2008; Cook et al. 2006) and the same degree of variation was found among powdery mildews infecting different host plant species/genera and belonging to as yet unresolved species complexes (e.g., Matsuda and Takamatsu 2003; Kiss et al. 2006; Takamatsu et al. 2008; Ito and Takamatsu 2010). Therefore, the accurate determination of the ITS sequences is essential for a precise identification of powdery mildew fungi, especially if morphological and/or host range data are limited. Also, accurate ITS sequences are needed for well-founded phylogenetic studies in the *Erysiphales*, especially when closely related species, including cryptic species (e.g., Kiss et al. 2006), are studied.

During direct sequencing of the ITS region in some powdery mildew samples, the electrophoregrams examined in our laboratory suggested some variation in the ITS sequences obtained. Therefore, the objective of this study was to determine whether there is any variation in the PCR-amplified ITS sequences obtained from closely related powdery mildew species. We chose the study of four species studied by Jankovics et al. (2008), and two other species studied by Kiss et al. (2008), because these were known to

differ from each other in only one to five, and one to four, nucleotides in their ITS sequences, respectively. These two recent studies, each on a set of closely related taxa, have also revealed differences in the host ranges, genome-wide diversity and/or morphology of the chosen powdery mildew species, therefore becoming ideal targets for the planned work.

Materials and methods

Fungal DNA samples

Table 1 lists the designations, host plant species, and other data for the powdery mildew taxa included in this work. Samples of mycelia were collected from either fresh infected leaves, obtained from the field, or dried leaves preserved as herbarium specimens. Fresh fungal material, mainly conidia, were collected from the infected leaves using sterilized artist brushes, while dried mycelia were scraped off the herbarium specimens with sterile razor blades. All the powdery mildew samples were collected with special care to avoid contamination from other powdery mildew sources. All were collected in eppendorf tubes and stored at −18°C until whole-cell DNA was extracted from them using a Qiagen DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany).

PCR amplification and sequencing of the ITS region

The ITS region was amplified using the ITS1F/ITS4 fungal-specific primer pair as described in Szentiványi et al. (2005) except that a high fidelity *Pfu* DNA polymerase (MBI Fermentas, Vilnius, Lithuania) was used instead of *Taq*. The amplicons were sequenced both directly and after cloning them into a vector system (see below). Each PCR amplification was carried out in parallel in three tubes under identical conditions and the three PCR products were mixed before further use. As described by Kovács et al. (2008), the errors that might be generated by DNA polymerases which mislead direct sequencing of PCR products could be practically eliminated by this procedure. This is because the possibility of a DNA polymerase error at the same nucleotide position in three identical PCRs, run in parallel, is almost zero. Thus, we applied this procedure and purified the amplicons with a PCR Clean up-M kit (Viogene, Hong-Kong, China), for direct sequencing of both strands done by LGC Genomics (Berlin, Germany) using ITS1F/ITS4 primers. Before cloning the purified amplicons into a pGEMT Easy Vector system (Promega, Madison, WI, USA), these were A-tailed using a normal *Taq* polymerase and dATP (MBI Fermentas, Vilnius, Lithuania) and purified again using the PCR Clean up-M kit. Subsequent steps of the cloning procedure were performed as described by Kovács et al. (2007). At least ten positive clones from each amplicon were sequenced by LGC Genomics using universal primers.

Data analyses

Sequences were compiled from electrophoregrams, aligned and checked as described in Kovács et al. (2008).

Results and discussion

Some of the electrophoregrams obtained from direct sequencing of the ITS region contained double peaks at certain positions thus revealing the presence of more than one ITS types with different nucleotides at those positions. In addition, shifts of the signs making the electrophoregrams useless upward from a certain nucleotide position on both-direction reads were also detected in some powdery mildew samples. These two signals revealed substitutional and indel events, respectively, in the directly sequenced ITS amplicons as shown in previous works (e.g., Smith et al. 2007). Sequences of the clones obtained from samples exhibiting variable nucleotide positions in the electrophoregrams of the directly sequenced ITS regions confirmed the presence of different ITS types in these fungi. These sequences contained different nucleotides in all the positions seen as variable sites in the electrophoregrams of the directly sequenced amplicons. Other nucleotide polymorphisms, not seen in the electrophoregrams of the directly sequenced ITS amplicons, were also detected in these samples.

Altogether, 17 samples of six powdery mildew species were studied (Table 1). Among these, four species, *O. longipes, O. neolycopersici*, an *Oidium* sp. ex *Chelidonium majus* and another *Oidium* sp. ex

Table 1 Designation, species identity, host plant, place and year of collection and herbarium accession number of each powdery mildew sample included in this study. GenBank accession numbers of the internal transcribed spacer (ITS) sequences of the nuclear ribosomal DNA (rDNA), determined after cloning the PCR-amplified ITS region, are also given. For each sample, identical ITS sequences were deposited in GenBank under a single accession number

Designation	Powdery mildew fungus	Host plant species	Place and year of collection	Herbarium accession number[a]	GenBank accession number of rDNA ITS sequences
Ch-1	*Oidium* sp.	*Chelidonium majus*	Martonvásár, Hungary, 2004	HAL 1842F	HQ286644, HQ286645
Ch-2	*Oidium* sp.	*C. majus*	Martonvásár, Hungary, 2005	HAL 2376F	HQ286646–HQ286648
Ch-3	*Oidium* sp.	*C. majus*	Budapest, Hungary, 2005		HQ286649–HQ286652
Ch-4	*Oidium* sp.	*C. majus*	Nitra, Slovakia, 2006	HAL 2377F	HQ286653–HQ286659
Ch-5	*Oidium* sp.	*C. majus*	Velčice, Slovakia, 2006	HAL 2378F	HQ286660–HQ286663
Ch-6	*Oidium* sp.	*C. majus*	Pardubice, Czech Republic, 2008	HAL 2379F	HQ286664, HQ286665
Ch-7	*Oidium* sp.	*C. majus*	Simpheropol, Ukraine, 2008	HAL 2380F	HQ286666–HQ286669
KTP-01	*O. neolycopersici*	*Solanum lycopersicum*	Nara, Japan, 2005		HQ286670–HQ286672
On-04	*O. neolycopersici*	*S. lycopersicum* cv. Brazil	Berre, France, 2005		HQ286685–HQ286688
On-USA-1	*O. neolycopersici*	*S. lycopersicum*	Riverhead, New York, USA, 2005		HQ286681–HQ286684
Pa-1	*Oidium* sp.	*Passiflora caerulea*	Wageningen, The Netherlands, 2005	HAL 2074F	HQ286689, HQ286690
Aq-2	*Erysiphe aquilegiae*	*Aquilegia vulgaris*	Debrecen, Hungary, 2006	HAL 2076F	HQ286643
OL-HU1	*O. longipes*	*Petunia* x *hybrida* cv. Wave Blue	Martonvásár, Hungary, 2006	HAL 2072F, BPI 878253	HQ286678, HQ286679
OL-US1	*O. longipes*	*P.* x *hybrida* cv. Surfinia Violet	Pompton Plains, NJ, USA, 2006	HAL 2071F, BPI 878251	HQ286680
OL-AT1	*O. longipes*	*P.* x *grandiflora*	Aspang, Austria, 2006	HAL 2070F, BPI 878252	HQ286675–HQ286677
Ol-1	*O. lycopersici*	*S. lycopersicum*	Tasmania, Australia, 1994	DAR 70008	HQ286673
Ol-2	*O. lycopersici*	*S. lycopersicum*	Victoria, Australia, 1980	DAR 35763	HQ286674

[a] HAL = Herbarium of Martin Luther University, Halle, Germany, BPI = U.S. National Fungus Collection, Beltsville, MD, USA, DAR = Plant Pathology Herbarium, Orange Agricultural Institute, Australia

Passiflora caerulea exhibited ITS nucleotide polymorphisms. All but one sample of these four species, 1, 3, 7 and 1, respectively, collected from distant places in Europe, USA and Japan (Table 1), contained variable nucleotide positions in their ITS regions (Table 2). In these samples two to seven different ITS sequence types were detected and these differed in up to three nucleotide positions (Table 2). Some of these were not seen on the electrophoregrams of the directly sequenced ITS amplicons. None of the samples of *E. aquilegiae* and *O. lycopersici* showed any variation in either the directly sequenced or cloned and sequenced ITS regions.

The detected variation in the ITS sequences of all but one sample representing *O. longipes, O. neolycopersici*, an *Oidium* sp. ex *C. majus* and another *Oidium* sp. ex *P. caerulea* was similar to the ITS nucleotide differences often described as distinguishing closely related powdery mildew species or other taxa such as formae speciales. For example, the ITS sequence published for a tree-parasitic species, *E. catalpae*, differed in only one or two nucleotides from those sequenced in some herb-parasitic species, such as *E. macleayae, E. circaeae* and *O. neolycopersici*, and did not differ at all from that of *E. aquilegiae* (Cook et al. 2006). In the same group of closely related fungi, Jankovics et al. (2008) showed that one to five nucleotide differences in the ITS sequences of *O. neolycopersici, E. aquilegiae* and powdery mildews identified as different *Oidium* spp. infecting *C. majus, P. caerulea* and *Sedum alboroseum*, respectively, are clearly in accordance with differences in the AFLP patterns and the host ranges of these plant pathogens. Similarly, distinct formae speciales of *Blumeria graminis*, infecting different monocot genera, including cereals, differ in a few nucleotide positions in their ITS sequences and also in their AFLP patterns and sequences of nuclear genes such as the beta-tubulin and the chitin synthase genes (Wyand and Brown 2003; Inuma et al. 2007). In other groups of the *Erysiphales* it was

Table 2 Number of clones sequenced, number of different ITS types detected and the maximum number of nucleotide positions with variable characters detected in each powdery mildew sample

Sample designation	Number of clones sequenced	Number of different ITS types	Maximum number of nucleotide positions with variable characters
Ch-1	11	2	1
Ch-2	20	3	2
Ch-3	11	4	2
Ch-4	10	7	3
Ch-5	11	4	2
Ch-6	10	2	1
Ch-7	11	4	2
KTP-01	10	3	2
On-04	11	4	2
On-USA-1	11	4	2
Pa-1	11	2	1
Aq-2	11	1	-
OL-HU1	10	2	1
OL-US1	10	1	–
OL-AT1	10	3	2
Ol-1	10	1	–
Ol-2	10	1	–

also repeatedly shown that one to a few nucleotide differences in the ITS sequences are characteristic to closely related powdery mildews collected from different host plant species and/or genera. This was found in powdery mildew fungi belonging to the genera *Podosphaera* (Ito and Takamatsu 2010), *Golovinomyces* (Matsuda and Takamatsu, 2003), *Sawadaea* (Hirose et al. 2005), *Pleochaeta* (Kiss et al. 2006), *Blumeria* (Inuma et al. 2007), *Phyllactinia* (Takamatsu et al. 2008) and so on, although in these genera the species identities are still not well-resolved and some taxa are considered as cryptic species (e.g., Kiss et al. 2006). In the *Podosphaera tridactyla* complex, for example, a few nucleotide differences in the ITS sequences corresponded to specialization to different *Prunus* spp. (Cunnington et al. 2005) while Leus et al. (2006) showed that one single nucleotide difference in the ITS sequences of *P. pannosa* isolates distinguished two pathotypes of this pathogen. On the other hand, a few nucleotide differences were found in the ITS sequences of some well-defined powdery mildew species; for example, such intra-specific differences were reported in samples of the oak pathogen *E. alphitoides* (Takamatsu et al. 2007) as well as in specimens of the newly described species *E. quercicola* infecting a number of oak species and unrelated tropical and subtropical tree species (Takamatsu et al. 2007).

Thus, a part of the published data, obtained using direct sequencing, suggested that even a divergence as low as a single nucleotide difference in the ITS sequences indicates differences in the host range patterns and/or at the genomic level in powdery mildew fungi. Such differences in the ITS sequences can, however, be detected even in a single sample of a powdery mildew fungus as revealed here. One to a few nucleotide difference in the ITS sequences cannot be used as an exclusive tool for the precise identification of a powdery mildew fungus at a species or a sub-species level. Such variation, in itself, is a valuable information but should be evaluated together, when available, with other data such as morphology, host range and genetic information obtained from the study of other nuclear or mitochondrial loci.

Sequencing following cloning can reveal most of the intrinsic variability of the ITS region in a DNA sample. Thus, in powdery mildew research, ITS sequences should be used as species or sub-species barcodes only if determined in this way, especially when closely related taxa are to be distinguished from each other. In rust fungi (the *Uredinales*), for example, cloning before sequencing the ITS region has already become a general protocol (Rossman 2007) because variations in the ITS sequences of individual samples were shown in many species (e.g., Alaei et al. 2009).

In two out of the six powdery mildew species included in this work, namely in *E. aquilegiae* and *O. lycopersici*, no ITS nucleotide polymorphisms were detected in any samples. In the other four species, such polymorphisms were found in all but one sample. This might indicate that some powdery mildew taxa are more variable at the ITS level than others but more species should be studied for well-founded conclusions to be drawn in this matter.

This work showed that direct sequencing can reveal the presence of different ITS sequence types in a sample especially if this was carefully prepared to reduce potential errors caused by DNA polymerases during PCR (Kovács et al. 2008). Samples exhibiting variable nucleotide positions in the chromatograms of

their directly sequenced ITS regions did contain different ITS sequence types identified by sequencing of the cloned amplicons while no variation was detected in any samples where chromatograms from direct sequencing did not show variable nucleotide positions.

In fact, variation in the ITS sequences of the same fungal samples might be more frequent than generally considered (Simon and Weiss 2008) especially because the signs indicating real variation could be ignored, being considered as background noise, DNA polymerase errors or simply overlooked. This is important to note because the ITS region is by far the most commonly determined DNA region in plant pathogenic and other fungi and it is routinely used for identification and DNA barcoding purposes (e.g., Bridge et al. 2003; Nilsson et al. 2008; Simon and Weiss 2008; Eberhardt 2010). A few nucleotide differences in the ITS region, thus a variation similar to what was detected in this work in single powdery mildew samples, is often considered as a reliable marker in the identification of certain fungal species (Bridge et al. 2003; Nilsson et al. 2008) especially if this is supported by sequences retrieved from GenBank. However, GenBank data have frequently been criticized for inaccuracies from several points of view (e.g., Bridge et al. 2003; Kang et al. 2010) including the means of obtaining the genetic information deposited in this database. This work showed that the ITS sequences determined in powdery mildew samples, and eventually deposited at GenBank, might not describe all the existing variation in this DNA region and the routine direct sequencing of the ITS region might mask diversity of these important pathogens.

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