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Characterisation and phylogeny of repeated elements giving rise to exceptional length of ITS2 in several downy mildew genera (Peronosporaceae)

Marco Thines *

University of Hohenheim, Institute of Botany 210, D-70593 Stuttgart, Germany

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Abstract

In downy mildews with pyriform haustoria (DMPH), ITS lengths between 1121 and 2587 kb were observed. The extreme length of ITS is due to sequence repeats of a poorly conserved part of the ITS2. Secondary structure analysis suggests that individual repeats may form long hairpin structures. The presence of these types of sequence repeats appears to be a synapomorphy for the DMPH. In another group of downy mildews, represented by *Hyaloperonospora*, similar REs sporadically occur, although with element lengths that are shorter than those in the DMPH group. Two characteristic motifs could be identified and their role in the evolution of the REs is briefly discussed. Dotplot analysis and molecular phylogenetic reconstructions for the repeated elements of representative species show distinct patterns that may suggest how they evolved. The high amount of sequence variability makes the repeated units a useful tool to study speciation and radiation processes in an economically important group of plant pathogens, which may form a model system for studying other coevolutionary host-pathogen systems.

Keywords: Phylogeny; Evolution; Oomycetes; Dotplot; ITS structure; DMPH

1. Introduction

The internal transcribed spacer (ITS) is located between the small and the large ribosomal subunits and is divided into two parts by the 5.8S gene, which lies between the ITS1 and the ITS2 regions. Recently, common secondary structures have been proposed for each of the ITS regions of even distantly related organisms (Gottschling and Plöttner, 2004; Joseph et al., 1999; Schultz et al., 2005), although the functional basis has not yet been elucidated. Although the ITS has been reported to have a regulatory function in ribosome genesis, the underlying mechanisms are unclear (van der Sande et al., 1992; van Nues et al., 1995; Smith and Steitz, 1997). From the time of the earliest molecular phylogenetic investigations, however, this region has been extensively used for phylogenetic analyses (Gonzalez et al., 1990). In general, the level of variability found in the ITS regions makes this region suitable for molecular phylogenetic reconstructions from family to subspecies level. For the downy mildews and closely related oomycetes, studies reporting ITS data have been conducted (Constantinescu and Fatehi, 2002; Cooke and Duncan, 1997; Cooke et al., 2000, 2002; Choi et al., 2003; Leclerc et al., 2000; Voglmayr, 2003; Thines and Spring, 2005), but often encountered levels of sequence variability that were either too high (Cooke et al., 2000) or rather low (Voglmayr, 2003). Therefore, reconstructions based on multiple loci of the nrLSU have been used (Göker et al., 2004; Voglmayr et al., 2004; Thines et al., 2006) to get a better resolution either on the backbone or the ultimate branches of the resulting trees, but there are still some aspects of the phylogeny of the downy mildews that have not been unequivocally resolved. Among

* Fax: +49 711 459 3355.
E-mail address: thines@uni-hohenheim.de

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these is the question of whether or not the downy mildews with pyriform haustoria (DMPH) form a monophyletic assemblage and from which other group of downy mildews they could have evolved. Recently, large repeated elements (REs) have been uncovered in the ITS2 region of *Plasmopara halstedii*, the downy mildew pathogen of sunflower (Thines et al., 2005a), and in some collections of *Hyaloperonospora parasitica* s.l. (Göker et al., 2004). Although such REs are not commonly found in all populations of *H. parasitica* and related species (Göker et al., 2004; Choi et al., 2003; Voglmayr, 2003), they are found consistently in all species of the Peronosporaceae with pyriform haustoria which have been examined (Thines et al., 2005b, Thines, unpublished results). The aim of this study was to determine the characteristics, evolution, and phylogeny of the REs, as well as to evaluate their usefulness for taxonomic investigations in the downy mildews and closely related genera.

2. Materials and methods

2.1. Peronosporomycete material

The Peronosporomycete (oomycete) material used for sequence analyses in this study and ITS sequences obtained from GenBank are summarised in Table 1. All specimens were air-dried before use, except for specimen 720 (*Protobremia sphaerosperma*), which was subjected to DNA extraction after deep freezing at −70 °C for 1 week. Sporangia and sporangiophores were harvested from the lower surface of infected leaves using a vacuum manifold, and then transferred to 2 ml tubes for DNA extraction.

2.2. DNA extraction, PCR, cloning and sequencing

About 2 mg of dried sporangia and sporangiophores were disrupted at room temperature using a mixer mill (Retsch, Germany) at 10 Hz for three minutes, adding one magnetic ball size 1/8″. DNA was extracted using the method of May and Ristaino (2004), modified as described in Thines and Spring (2005). The ITS region was amplified using the oomycete specific primer ITS1-O (Table 2; Bachofer, 2004), which contains the conserved starting motive of ITS-1, 18S_rc (reverse complementary to 18SR Medlin et al., 1988) and LR-0 (reverse complementary to LR-0R Moncalvo et al., 1995). PCR was carried out in an Eppendorf Mastercycler (Eppendorf, Germany) using the following conditions: denaturation at 94 °C for 42 s, annealing at 56 °C for 42 s, elongation for 1 min 42 s at 72 °C. Cycles were repeated 30 times and succeeded by a final elongation step of 4 min 20 s. Amplification resulted in single bright fragments for all samples investigated. The amplified fragments were cut from the gel and cleaned, using the QiaQuick PCR-Purification Kit (Qiagen, Germany). The cleaned fragments were cloned into the *Escherichia coli* strain supplied with the TOPO PCR cloning kit (Invitrogen, Germany), following the manufacturer's instructions. Colonies were grown on selective media for

Table 1
Peronosporomycete material and sequences obtained from GenBank used for sequence analyses

Species	Host	Collection details/accession number	Deposited in
Bremia lactucae s.l.	*Cirsium arvense*	Germany, Stuttgart, Hohenheim; 19. Sept. 2001; leg. Otmar Spring. DQ665664	HUH 429
Hyaloperonospora parasitica a	*Helianthemum* sp.	Germany, Wittenberg, OBI; 12. Apr. 2004; leg. Marco Thines. DQ665665	HUH 682
Peronospora arborescens	*Papaver somniferum*	Laboratory strain. Internal handling number Pa1-05.	HUH 770
Plasmopara halstedii	*Helianthus annuus*	Laboratory strain. Internal handling number Ph1-00. DQ665670	HUH 114
Plasmopara obducens	*Impatiens balsamina*	PR China, Yunnan, Kunming, YAU; 27. Aug. 2004; leg. Marco Thines. DQ665666	HUH 650
Plasmopara pusilla	*Geranium pratense*	Germany, Tübingen, Steinenberg; 05. May 2002; leg. Otmar Spring. DQ665667	HUH 453
Plasmopara viticola	*Vitis vinifera*	Germany, Esslingen, Mettingen; 05. Aug. 2002; leg. Otmar Spring. DQ665668	HUH 491
Protobremia sphaerosperma	*Tragopogon orientalis*	Germany, Schwäbische Alb, R 351448, H 536057; leg. Marco Thines. DQ665669	HUH 720
Halophytophthora batemanensis		AF271223	GenBank
Hyaloperonospora parasitica b		AY531452	GenBank
Peronophythora litchi		AY251666	GenBank
Phytophthora cactorum		AB217673	GenBank
Phytophthora infestans		AF266779	GenBank
Pythium monospermum		AY598621	GenBank

HUH, Herbarium of the University of Hohenheim.

Table 2
PCR and sequencing primers used in this study

Primer name	Primer sequence (5′–3′)	Target organism
ITS1-O	CGG AAG GAT CAT TAC CAC	Peronosporomycetes
LR-0	GCT TAA GTT CAG CGG GT	Peronosporomycetes
18S_rc	GTA GGT GAA CCT GCA GAA GGA TCA A	Peronosporomycetes
Seq_Pl_ITS2-F	ACT GTA CTT CTC TTT GCT C	*Plasmopara*
Seq_Pl-vit_ITS2-F	TGG TGG GTC GTA GTT ACG ACG	*Plasmopara viticola*
Seq_Br_ITS2-F	AGC CGA AGC CAA CCA TAC C	*Bremia*
Seq_ITS2-F	GCC AGA TGT GAA GTG TCT TGC G	DMPH
Seq_ITS2-R	AAA GCT GCC ACT CTA CTT CG	DMPH