

Characterization of the O.ul-mS952 Intron: A Potential Molecular Marker to Distinguish Between *Ophiostoma Ulmi* and *Ophiostoma Novo-Ulmi* Subsp. *Americana*

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Abstract—The full length mitochondrial small subunit ribosomal (mt-*rns*) gene has been characterized for *Ophiostoma novo-ulmi* subspecies *americana*. The gene was also characterized for *Ophiostoma ulmi* and a group II intron was noted in the mt-*rns* gene of *O. ulmi*. The insertion in the mt-*rns* gene is at position S952 and it is a group IIB1 intron that encodes a double motif LAGLIDADG homing endonuclease from an open reading frame located within a loop of domain III. Secondary structure models for the mt-*rns* RNA of *O. novo-ulmi* subsp. *americana* and *O. ulmi* were generated to place the intron within the context of the ribosomal RNA. The *in vivo* splicing of the O.ul-mS952 group II intron was confirmed with reverse transcription-PCR. A survey of 182 strains of Dutch Elm Diseases causing agents showed that the mS952 intron was absent in what is considered to be the more aggressive species *O. novo-ulmi* but present in strains of the less aggressive *O. ulmi*. This observation suggests that the O.ul-mS952 intron can be used as a PCR-based molecular marker to discriminate between *O. ulmi* and *O. novo-ulmi* subsp. *americana*.

Keywords—Dutch Elm Disease, group II introns, mtDNA, species identification

I. INTRODUCTION

DUTCH Elm Disease (DED) is a fungal disease that has devastated many urban forests that contain *Ulmus americanus* L. (American elm) and related species. The causative agents of DED are *Ophiostoma ulmi* (Buism.) Nannf., and subspecies of *Ophiostoma novo-ulmi* Brasier. These fungi are filamentous ascomycetes microfungi belonging to the Order Ophiostomatales, Family Ophiostomataceae.

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These fungi are transmitted with the help of bark beetles (Family Curculionidae, Subfamily Scolytinae). DED is a vascular wilt diseases caused by the DED fungi blocking the conductive tissue of the elm tree, thus preventing the flow of nutrients and water, thereby killing the tree.

Historically there have been two epidemics of DED, the first caused by *O. ulmi* and the current pandemic of this disease is caused by *O. novo-ulmi* [1]. *Ophiostoma novo-ulmi* based on morphological, physiological and molecular (in both nuclear and mitochondrial genomes) differences, has been segregated into *O. novo-ulmi* subsp. *novo-ulmi* (also known as the Eurasian race, EAN) and *O. novo-ulmi* subsp. *americana* (also known as the North American race, NAN) [2 - 4].

Several methods have been proposed to differentiate among isolates of these three biological forms (*O. ulmi*, *O. novo-ulmi* subsp. *novo-ulmi* and subsp. *americana*) such as colony morphology [5], pathogenicity [6], optimal growth temperature [7], soluble protein patterns [8], [9], and fertility/genetic barriers [1], [2], [5], [6]. Differentiating among the various DED causing agents by the above-mentioned criteria is time consuming. Due to advances in molecular biology techniques, large numbers of highly informative DNA markers have been developed for the detection of genetic polymorphism that allows for species and/or strain designations [10].

So far several molecular markers have been developed for the DED causing agents such as PCR based markers based on the nuclear *cerato-ulmi* (*cu*) and the colony type (*col1*) genes [4], however to differentiate between the two subspecies of *O. novo-ulmi* the PCR products have to be digested with restriction enzymes. Random amplified polymorphic DNA (RAPD) represents another PCR based technique that has been used to differentiate strains of *O. ulmi* and subspecies of *O. novo-ulmi* [11]. Restriction fragment length polymorphism (RFLP) of nuclear and mitochondrial DNAs has also been applied in analyzing populations of *O. novo-ulmi* and related taxa [12], [13].

Previously it has been shown that the size of the mtDNA might be useful for differentiating among subspecies of *O. novo-ulmi*; in general sizes of the mtDNAs for isolates of *O. novo-ulmi* subsp. *novo-ulmi* were shown to be significantly

larger than those for isolates of the more aggressive *O. novo-ulmi* subsp. *americana* [12 - 14]. In general it is thought that size variation among mtDNAs is due the sizes of intergenic spacers and (reviewed in [15], [16]).

Mitochondrial introns usually belong to either the group I or group II category of self-splicing introns and they tend to encode proteins that can assist in intron mobility or intron splicing or in some cases the encoded protein can served both functions [17].

The objective of this study is to characterize an intron located within the mt-*rns* gene and to evaluate if this intron (referred to as O.ul-mS952) could be used as a molecular marker that allows for the differentiation between strains of *O. ulmi* and *O. novo-ulmi* subsp. *americana*. A PCR-based method is presented that allows for the detection of the presence or absence of the O.ul-mS952 intron using a combination of exon-specific and intron-specific primers.

II. MATERIALS AND METHODS

A. Fungal Strains, Growth Conditions and DNA Extraction

The fungal strains used in this study, their sources and geographical origins are listed reference [18] 1. For routine culturing the strains were maintained in Petri plates containing 2% Malt Extract Agar (MEA) supplemented with 1 gl-1 yeast extract (YE) and 20 gl-1 bacteriological agar. From these cultures, agar plugs were cut containing fungal growths which were used to inoculate 250 ml conical flasks containing 50 ml PYG liquid medium (1 gl-1 peptone, 1 gl-1 yeast extract and 3 gl-1 glucose). Liquid cultures were incubated at 20 oC for 7 days to generate biomass for DNA or RNA extractions. After the incubation period the mycelium was harvested by filtration and then the mycelium was homogenized by vortexing with glass beads (1-3 mm, Fisher Scientific Canada, Ottawa, Ontario). The nucleic acids were extracted as described previously [19].

B. Amplification of mt-*rns* Gene

The mt-*rns* gene was amplified via PCR from 182 strain of DED fungi as previously described in Mullineux et al. [20] with the primers mtsr-1 and mtsr-2 (see Table 2 for a complete list of the primers used in the current study) utilizing the Invitrogen PCR system (Invitrogen, Burlington, Canada). The 182 strains analyzed include *O. ulmi* (11 strains), *O. novo-ulmi* subsp. *novo-ulmi* (1 strain) and *O. novo-ulmi* subsp. *americana* (170 strain) collected from different geographic regions (Canada, USA, England, Netherlands and Iran). Species designations were based on morphological and molecular data [16], [18]. The PCR conditions for the mtsr-1 and mtsr-2 primers were as follow: an initial denaturation at 93 °C for 1 minute followed by 25 cycles of denaturation (93 °C for 1 minute), annealing (55 °C for 1 minute) and extension (70 °C for 5 minutes) followed by a final extension step at 70 °C for 10 minutes.

A detailed PCR analysis for the presence of the O.ul-mS952 intron was performed on genomic DNAs from *O. ulmi* strain

TABLE I
A LIST OF THE PRIMERS USED IN PRESENT STUDY

Primer	Sequence (5' → 3')
M13-F	GTAACACGACGGCCAG
T7	ATTAACCCCTCACTAAAGGGA
rns-F	GAGTTTGGTGATGGCTCTGATTGAACACTG
mtsr-1	AGTGGTGACAGGTGAG
mS952-F	TTTGACACCAGTAGTGAAGTATG
rnsulmi-F3	GGATTAGAGACCCTTGTAGTCTTAGCAG
rnsulmi-F4	CTTTAAAGCGCTACCGTCTTGATTC
rnsulmi-F5	GCCCCAGGGCGAAAGGAAAATTAGTTA
rnsulmi-F6	GTGGTTTAGTAGACGCAGGAAGGAAG
M13-R	CAGGAAACAGCTATGAC
T3	TAATACGACTCACTATAGGG
rns-R	CCACTACACGAACCGTATTTCGAC
mtsr-2	CGAGTGGTTAGTACCAATCC
mS952DV-R	AACCGTACGTGCAACTTTCATCGCATACGGC
rnsulmi-R6	ACGTGCAACTTTCATCGCATACGGCTC
rnsulmi-R5	CTCTTTTTTAAACTTTTCAGATAAACC
rnsulmi-R4	ATTAGGCCATCTAATGTTAAGTGTTC
rnsulmi-R3	CTACGCTAGGGTTATGATAAATATTAC

DAOM 171046 and *O. novo-ulmi* subsp. *americana* strain DED 02-10 using combinations of exon-specific primers (mtsr-1, mS952-F and mtsr-2) and a intron-specific primer (mS952DV-R). The primers rns-F and rns-R were designed based on sequences that flank the mt-*rns* gene and these were used to amplify the complete sequence of the mt-*rns* gene for *O. ulmi* DAOM 171046 and *O. novo-ulmi* subsp. *americana* DED 02-10. The rns-F and rns-R primer set allowed for the sequence characterization of the entire mt-*rns* gene.

All PCR amplicons were analyzed by gel electrophoresis through 1% agarose gel in TBE buffer (89 mM Tris-borate, 10 mM EDTA, pH 8.0). The DNA fragments were sized using the 1-kb plus ladder (Invitrogen), and nucleic acids were visualized by staining with ethidium bromide (0.5 µg/ml) and exposing the stained gels with UV light.

C. Cloning and Sequencing

Ophiostoma ulmi strain DAOM 171046, *O. novo-ulmi* subsp. *americana* strain DED 02-10 and *O. novo-ulmi* subsp. *novo-ulmi* strain IMI 343.101 were selected for sequence characterization. The mt-*rns* derived PCR products were converted into sequencing templates by purifying them with the Wizard® SV Gel and PCR Clean-Up system (Promega, Madison, USA). The double stranded DNA fragments were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturers instructions. The sequencing products were denatured and resolved on a 3130 genetic analyzer (Applied Biosystems).

In some instances sequencing results were more consistent by working with cloned mt-*rns* derived PCR products. The PCR products were cloned into *Escherichia coli* (DH5α) using the TOPO TA Cloning® kit (Invitrogen). Recombinant plasmids were purified with the Wizard® Plus Minipreps DNA purification system (Promega). Initially, vector based primers as supplied by the TOPO cloning kit: M13 Forward, M13 Reverse, T7 (forward), and T3 (reverse) were used to obtain

primary sequences, thereafter primers were designed as needed to complete all sequences in both directions (TABLE I).

D. Sequence and Phylogenetic Analysis

Individual sequences were compiled and assembled manually into contigs using the GeneDoc program v2.5.010 [21] and nucleotide sequence alignments were done with the Clustal-X program [22]. Alignments were adjusted by eye with the GeneDoc program. The ORF finder program (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>; Genetic code setting for molds #4) was used to search for potential ORFs within the O.ul-mS952 intron.

For phylogenetic analysis sequences were obtained from NCBI and aligned with the aid of Clustal-X. The MrBayes program (MrBayes program v3.1 [23]) was used for generating the phylogenetic tree and the parameters for analyzing the nucleotide sequence alignment was as follows: GTR model with gamma distribution and four chains were run simultaneously for 1 000 000 generation with a sample frequency of 100 and a "burn-in" corresponding to the first 25% of sampled trees.

E. Intron Nomenclature and Secondary Structure Models

For naming introns we followed the nomenclature system proposed by Johansen and Haugen [24]; intron insertion sites are based on corresponding nucleotide positions within the *E. coli* SSU-rRNA sequence (GenBank accession AB035922). Based on this system the mt-*rns* intron was named O.ul-mS952 (O.ul = *O. ulmi*, mS = mitochondrial SSU-rRNA gene, and 952 = the insertion site with respect to the *E. coli* SSU-rRNA sequence).

The web server RNAweasel (<http://megasun.bch.umontreal.ca/RNAweasel/>; [25], [26]) was used to predict the intron class and signature folds within the intron. The online program *mfold* (<http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi>; [27]) was used to generate the final fold for the intron taking into account output from RNAweasel and features characterized for Group II introns in publications by Michel & Ferat [28] and Toor *et al.* [29]. The secondary structures for the complete small subunit RNA for *O. ulmi* (DAOM 171046) and *O. novo-ulmi* subsp. *americana* (DED 02-10) were generated by comparative sequence analysis with the SSU rRNA secondary structures of *E. coli* and *Aspergillus nidulans* (GenBank accession J01393). The final secondary structures for the intron and the mt-*rns* rRNA were drawn with CorelDraw™.

F. RNA Extraction and Reverse Transcription-PCR (RT-PCR)

For RNA extraction, *O. ulmi* DAOM 171046 was grown in 250 ml conical flasks containing 50 ml peptone yeast-extract glucose (PYG) liquid medium for up to 7 days at 20 °C. Fungal biomass was collected by vacuum filtration using Whatman number 1 filter paper and the wet mycelium was

flash-frozen in liquid nitrogen and ground to a fine powder using a precooled mortar and pestle. The RNA was extracted using the RNeasy® mini kit (QIAGEN, Mississauga, ON, Canada) following the manufacturer's procedures. In addition to the DNase step within the RNeasy® mini kit procedure the TURBO™ DNase kit (Ambion, Austin, TX, USA) was also applied according to the manufacturer's protocol to ensure the removal of all DNA from the RNA preparation. The TURBO™ DNase was inactivated by heating at 75 °C for 10 min. The ThermoScript™ RT-PCR system (Invitrogen) was used to synthesize cDNA using approximately 100 ng of template RNA. First strand synthesis was carried out with 40 pmol of mtsr-2 primer and subsequent PCR amplification was carried out with mtsr-1 and mtsr-2 primers both at 40 pmol per reaction. The PCR products generated by the RT-PCR reactions were sequenced as described previously.

III. RESULTS

A. The mt-*rns* Gene of *O. ulmi* and *O. novo-ulmi* subsp. *americana*

Fig. 1 (a) represents a schematic overview of the mt-*rns* gene, including the various primer binding sites that were developed for amplifying the mt-*rns* gene and the mS952 intron. For screening the 182 strains of DED fungi, the mt-*rns* gene was amplified using the primer mtsr-1 and mtsr-2 and the PCR products ranged in size between 1.2 kb and 3.0 kb [Fig 1 (b)]. A 1.2 kb PCR product is expected when no insertions are present within this gene; however, a 3.0 kb PCR product is indicative of insertions (introns). The *O. ulmi* strain DAOM 171046 yielded a 3079 bp PCR fragment (GenBank accession HQ292075), while the *O. novo-ulmi* subsp. *americana* DED 02-10 (GenBank accession HQ292074) yielded a 1213 bp PCR amplicon [Fig. 1 (b)]. The *O. novo-ulmi* subsp. *novo-ulmi* (GenBank accession JF837329) also yielded a 1212 bp PCR amplicon. Note sizes of the PCR products were determined by sequence analysis of representative strains. Based on comparing mt-*rns* sequences that lacked insertions with those that do, the intron was found to be inserted at the position mS952 with respect to the SSU-rRNA gene of *E. coli*. The intron-exon junctions were first determined by comparative sequence analysis of *O. ulmi* and *O. novo-ulmi* mtsr-1 and mtsr-2 mt-*rns* gene sequences [Fig. 1 (c)].

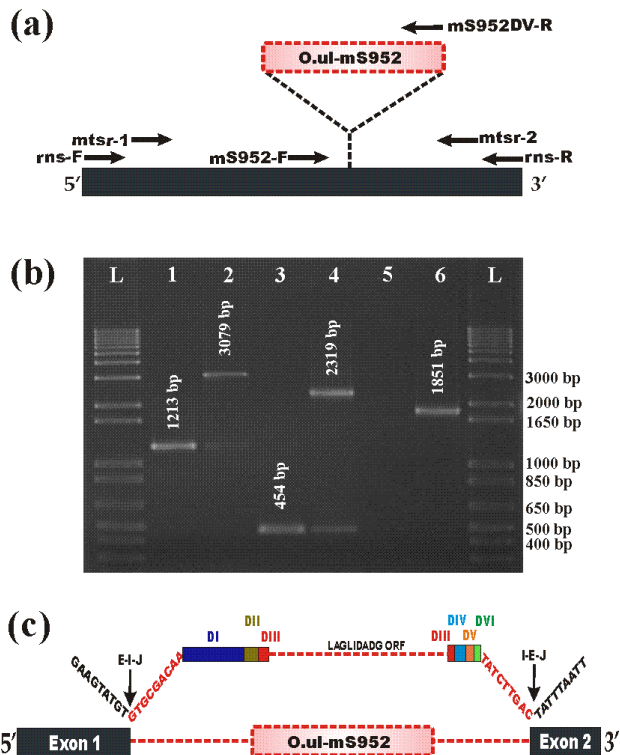


Fig. 1 (a) A schematic representation of the mt-rns gene showing the binding sites and directions of the primers used in this study; the dotted triangle represent the position of the O.ul-mS952 intron. (b) PCR amplicons of the mt-rns gene from *O. novo-ulmi* subsp. *americana* and *O. ulmi* (Lane 1 and 2 respectively) using the primer pair mtsr-1 and mtsr-2. The presence of O.ul-mS952 intron was checked using exon-specific primers flanking the intron insertion position, mS952-F and mtsr-2, which generate a 454 bp PCR fragment in the case of *O. novo-ulmi* subsp. *americana* (Lane 3) and a 2319 bp PCR fragment for *O. ulmi* (Lane 4). There appears to be evidence that there is mtDNA heterogeneity within *O. ulmi* as two bands were observed, the expected 2.3 kb fragment for the intron containing mt-rns allele but there is also evidence for the presence of an intron-less alleles revealed by the generation of the 454 bp PCR product (see lane 4). The absence (Lane 5) and the presence (Lane 6) of the O.ul-mS952 intron in *O. novo-ulmi* subsp. *americana* and *O. ulmi* respectively have been confirmed when the exon-specific primer mS952-F is combined with the intron-specific primer mS952DV-R. Sizes of the PCR products are indicated (sizes based on sequence determination). The L lane contains the resolved 1 kb plus ladder DNA fragments (Invitrogen). (c) A schematic overview of the *O. minus* mt-rns gene of *O. ulmi* showing the O.ul-mS952 group II intron which consisting of six domains (DI to DVI). The dotted line in DIII represents the LAGLIDADG homing endonuclease ORF. The upstream and downstream exon/intron junction (E-I-J) and the intron-exon junction (I-E-J) sequences respectively are indicated

Using internal exon-specific primers (mS952-F and mtsr-2) flanking the O.ul-mS952 intron for *O. ulmi* and *O. novo-ulmi* subsp. *americana* generated 2319 and 454 bp fragments respectively. These results confirmed the mtsr-1/mtsr-2 PCR survey results which were that the O.ul-mS952 intron is

present in all *O. ulmi* strains and is absent in all *O. novo-ulmi* strains. A combination of exon-specific primer (mS952-F) and intron-specific primer (mS952DV-R) generate a 1851 bp PCR fragment for *O. ulmi* strains, while no amplicons were generated for *O. novo-ulmi* strain [Fig. 1 (b)].

B. *O.ul171046-mS952 Intron*

Group II introns have conserved secondary structures at the RNA level, that can be visualized as six stem-loop domains (domains I to VI) emerging from a central wheel [28]. The O.ul-mS952 intron is a typical group II intron [Fig. 2] containing the characteristic features of class B1: such as the exon binding sites (EBS1, EBS2 & EBS3) which are complementary to the intron binding sites (IBS1, IBS2 & IBS3) in the upstream and the downstream exons flanking the intron insertion site; the internal loop of DICi (ϵ), the internal loop within DII, the linkers between domains I to VI and the absence of insertions in the 3' strand of DIi and DIi [28], [29]. The ORF finder program identified an ORF within the O.ul-mS952, this ORF encodes a potential double motif LAGLIDADG homing endonuclease rather than the reverse transcriptase-ORFs that typically are associated with group II introns. Folding the intron RNA indicates that the location of the LAGLIDADG ORF is within domain III [Fig. 2].

C. *Ophiostoma ulmi* mt-rns RNA Secondary Structure Model

Fig. 3 provides a secondary structure model for the mt-rns RNA consisting of four well-defined domains denoted I, II, III & IV [30, 31]. The O.ul-mS952 intron insertion site was characterized within this secondary structure model and the intron is located in a stem region at the lower half of domain III of the 16S rRNA molecule.

D. *In vivo* Splicing of the O.ul-mS952Intron

Total RNA was isolated from the *O. ulmi* strain DAOM 171046 and analyzed by RT-PCR, as a means of determining the *in vivo* splicing activity of the O.ul-mS952 intron [Fig. 4]. Genomic DNA of *O. ulmi* DAOM 171046 was used as a control for the RT-PCR experiments. The DNA amplification of the mt-rns gene from the genomic DNA was carried out using the mtsr-1 and mtsr-2 primers. Standard PCR yielded a product of 3081 bp representing the mt-rns exon sequence (1211 bp) as well as the O.ul-mS952 intron sequence (1868 bp). The mtsr-1/mtsr-2 amplification using cDNA as a template resulted in a 1211 bp fragment; the later is expected if the O.ul-mS952 has been spliced out. Sequence analysis of the cDNA confirmed that the intron was indeed spliced out, the sequence data also confirmed the predicted exon/intron junction sequences previously based on comparative sequence analysis [Fig. 1 (c)].

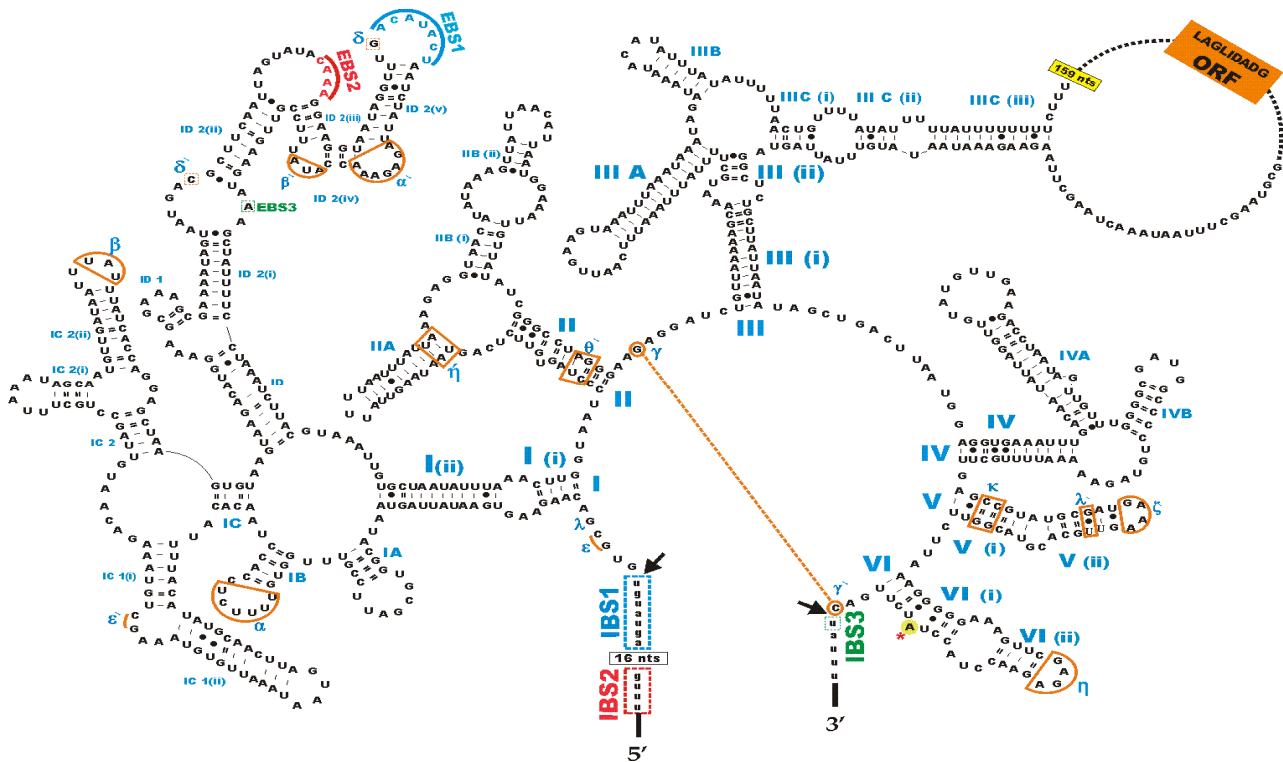


Fig. 2 The secondary structure of the *O.ulmS952* group II B1 intron RNA. Intron sequences are in upper-case letters and exon sequences are in lower-case letters. The positions of EBS1, EBS2 and EBS3 are noted. The positions of IBS1, IBS2 and IBS3 in the 5' and 3' exons are boxed with dotted lines. Tertiary interactions are indicated by dashed lines and Greek letters (ϵ , λ , α , β , θ , κ , ζ , and γ). The six major structural domains are indicated by Roman numbers (I, II, III, IV, V and VI). The solid black arrows indicate the intron-exon junctions (5' and 3' splicing sites). The asterisk shows the bulged adenosine nucleotide in domain VI (the branch point). The LAGLIDADG ORF is encoded within DIII

E. The mS952 Intron ORF Family

Phylogenetic analysis of mS952 intron ORF sequences showed that the *O. ulmi* mS952 intron ORF sequence is related to sequences found in species that belong to the related asexual genus *Leptographium* [Fig. 5]. Using the most distantly related sequence from *Cryphonectria parasitica* as the outgroup showed that the *O. ulmi* sequence is positioned at the basal node from which all the *Leptographium* mS952 ORF sequences can be derived. This is expected as *Ophiostoma* is a genus that is closely related to *Grosmannia* (a sexual genus that is defined by forming asexual reproductive structures that can be assigned to *Leptographium*) [32]. Based on the currently available sequences there is no strong evidence for horizontal movement of the mS952 intron within the ophiostomatoid fungi.

IV. DISCUSSION

A. The mS952 Intron Distinguishes Between Strains of *O. ulmi* and *O. novo-ulmi* subsp. *americana*

Ophiostoma ulmi is viewed as the first recognized DED causing agent that was introduced into North America in the late 1930s (reviewed in [33]). More recently in North America *O. ulmi* appears to have been replaced by *O. novo-ulmi* subsp. *americana* but in Europe and Asia all three forms of the DED causing agents can still be found [34]. Although these fungi are viewed as distinct taxa they seem to be able to mate under certain conditions and hybrids have been recovered from various locations in Europe, the later is of concern as hybridization between these species/subspecies might be one mechanisms whereby more virulent forms of DED causing agents can arise [4], [33].

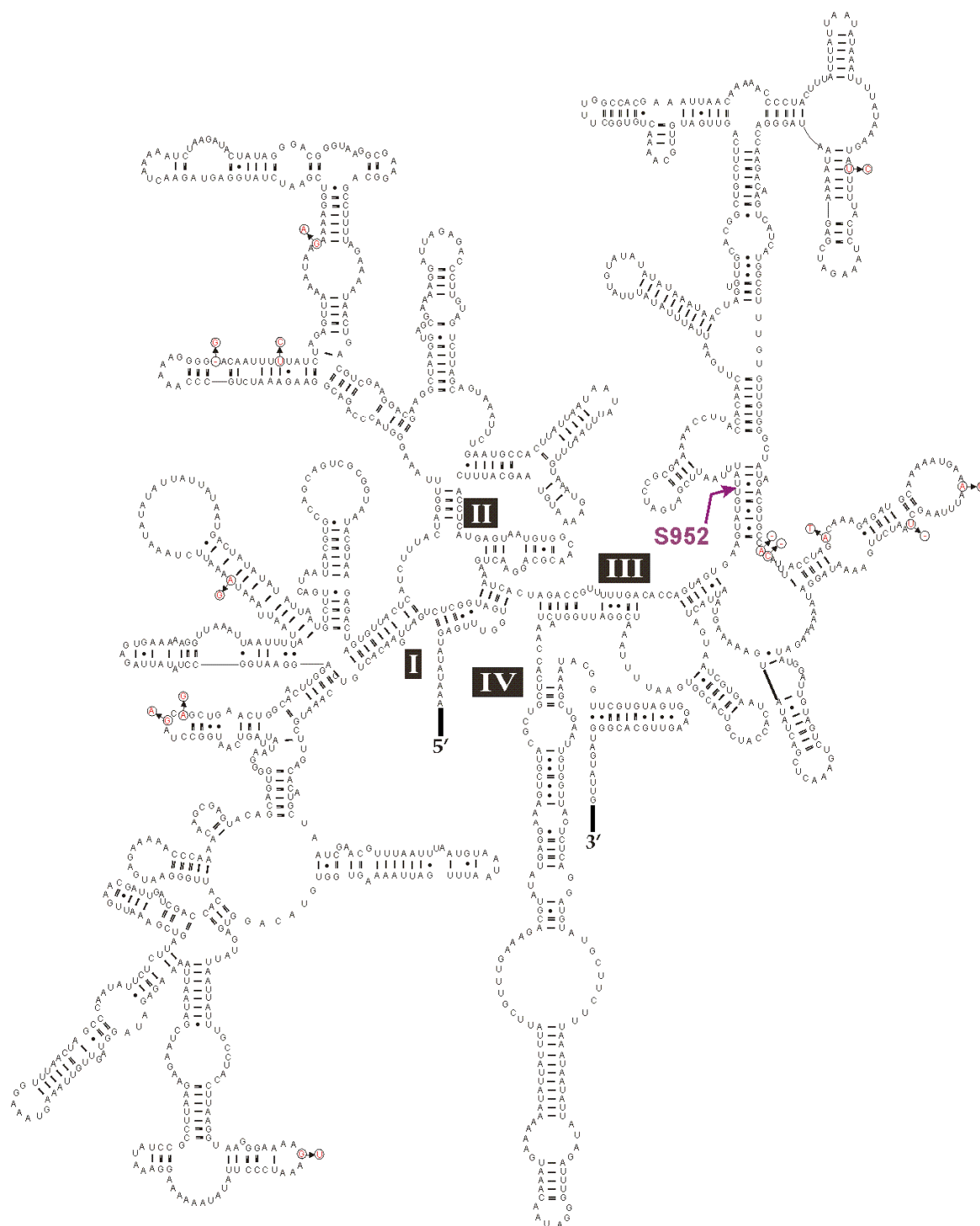


Fig. 3 A secondary structure model for the mt-rns RNA of *O. novo-ulmi* subsp. *americana* showing the four structural domains (I to IV). The circled nucleotides indicates the differences between *O. novo-ulmi* subsp. *americana* and the *O. ulmi* mt-rns RNA. The O.ul-mS952 intron insertion site is also indicated and it is located in a stem region in domain III

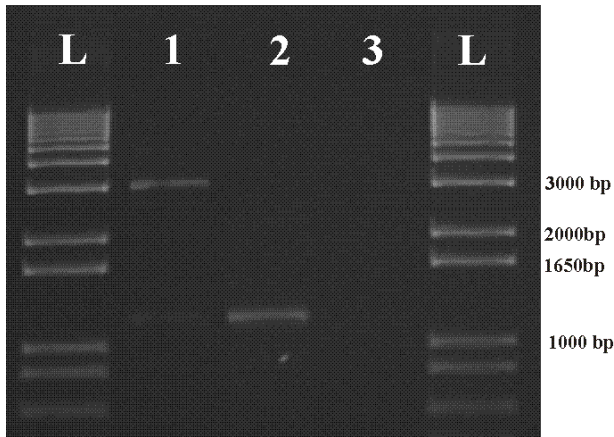


Fig. 4 RT-PCR analysis for demonstrating the *in vivo* splicing of the *O. ulmi*-mS952 intron. The mt-*rns* transcripts for the *O. ulmi* DAOM171046 was analyzed by RT-PCR. The standard PCR reaction using genomic DNA as a template is shown in lanes 1 and it generated a 3.0 kb fragment. The amplicon length for the cDNA was 1.2 kb (Lane 2) indicating that the *O. ulmi*-mS952 intron was splicing out. The negative controls for RT-PCR (standard PCR using DNAs free whole cell RNA as a template without the RT step) assay yielded no bands (lane 3) showing that the RNA was free of any DNA. The lane marked "L" contains the 1 kb plus ladder (Invitrogen)

Indeed it is proposed that *O. novo-ulmi* itself might be a product of a hybridization event between *O. ulmi* and another closely related *Ophiostoma* species [35].

Insertions or deletions of introns within the mtDNA genes are probably the major source of DNA based polymorphisms between *O. ulmi* and the *O. novo-ulmi* subspecies [14], [16]. Due to sequence conservation of the mtDNA genes PCR based primers can be readily designed to allow for surveying genes for the presence of optional introns. The goal of this work was to characterize an mt-*rns* intron and to evaluate if this intron is stable enough to be a potential marker that could be useful for distinguishing among the fungal agents of DED.

Although we assayed for the presence or absence of introns using a PCR approach [see Fig. 1 (a)] we have to admit that a "minus" result does not unambiguously identify taxa that lack introns. Each mitochondrion can contain many copies of the mt chromosome. PCR potentially amplifies smaller DNA fragments thus if there is heterogeneity among the mtDNAs rare intron-plus alleles could be missed. Therefore we utilized one primer combination (mS952-F and mS952DV-R) where the forward primer is exon based and the reverse primer is intron based. This combination should capture instances where an intron containing mt-*rns* gene was missed as its PCR derived product might be outcompeted by intron-less mt-*rns* PCR derived product as the shorter fragments could be amplified preferentially when using exon based primers.

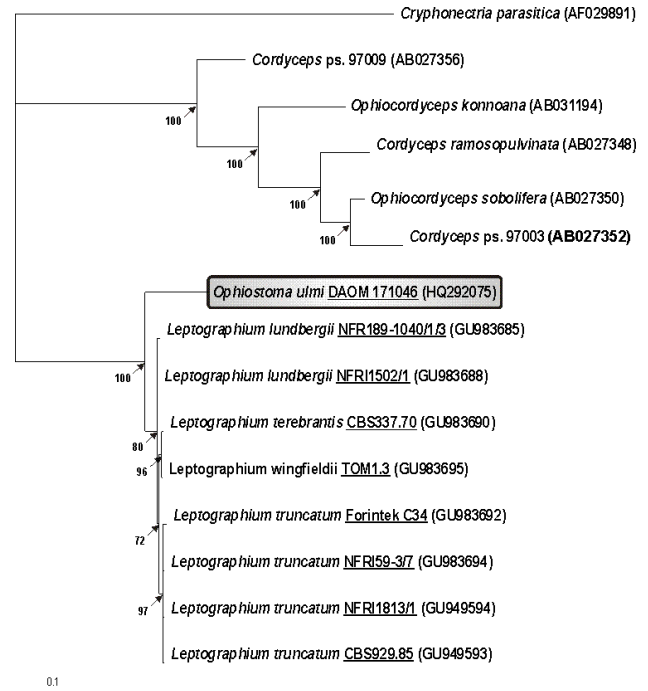


Fig. 5 Phylogenetic tree showing the phylogenetic position of the *O. ulmi* mS952 group II intron ORF nucleotide sequence within the mS952 intron ORF family. The tree includes sequences from *Leptographium* and *Cordyceps* spp., and the tree is rooted with the *C. parasitica* mS952 intron ORF sequence. The tree topology is based on Bayesian analysis and the numbers at the nodes represent the % posterior probability values as obtained from a 50% majority rule Bayesian consensus tree. The branch lengths are based on Bayesian analysis and are proportional to the number of substitutions per site. Strain numbers are underlined, and the Genbank sequence accession numbers are listed in brackets.

There appears to be evidence that there is mtDNA heterogeneity within *O. ulmi* as two bands were observed when exon based primers was used [Fig. 1(b)], however we never noted evidence for additional bands within *O. novo-ulmi* subsp. *americana*. In addition the primer combination: mS952-F and mS952DV-R failed to detect any evidence for the presence of introns within strains of *O. novo-ulmi* subsp. *americana*. So we can state that the mS952 intron is found in *O. ulmi* but not in *O. novo-ulmi* subsp. *americana*.

The absence of the intron in all *O. novo-ulmi* subsp. *americana* strains suggests that this intron could be a useful marker in distinguishing these two economically important species. In areas where both species still exist a molecular marker might be an alternative to cultural methodologies for distinguishing these two species. Due to the potential for the various DED agents to hybridize monitoring the presence of *O. ulmi* might be valuable in risk assessment for predicting future pandemic of DED. In experimental approaches where these fungi are maintained in the same laboratory or where hybrids might have to be generated the identified molecular

markers might be very valuable in facilitating the analysis of inter-species crosses or heterokaryon formation.

In a previous study on the mtDNA large ribosomal subunit gene (*rnl*) gene [20], we noted that there was evidence that the *rnl*-U7 group I intron (=mL1699) could potentially be transferred between *O. novo-ulmi* subsp. *americana* and *O. ulmi*. This demonstrates that group I introns like group II introns are mobile elements that can readily cross species barriers, in particular between closely related species such as members of the *O. ulmi* species complex. However, we failed to detect any mS952 introns within *O. novo-ulmi* subsp. *americana* suggesting that this intron so far failed to be transferred between the two species. It is known that for efficient splicing both group I and II introns usually required host genome encoded splicing factors/maturases and one could speculate that the mS952 intron requires a factor for efficient splicing that is not available within the *O. novo-ulmi* subsp. *americana* genetic background. In order for a potentially mobile element to move horizontally to a new species it has to find a compatible genomic environment that allows the element to be non-toxic to the new host. Splicing deficiency could be costly to the host, for example in a recent study on a mtDNA *rns* group II intron in *Cryphonectria parasitica* (Chestnut blight fungus) splicing deficiency was linked to growth abnormalities and hypovirulence [36].

B. The 16S rRNA Secondary Structure Model and the *O.ul*-mS952 Intron

Sequence characterization has shown that the *O. ulmi* mt-*rns* intron is a group II intron that encodes a LAGLIDAG type ORF in domain III. Similar introns have been previously described and they are novel as typically group II introns encode reverse transcriptase-like ORFs not homing endonucleases [19], [37], [38]. The RT-PCR results confirm that this element is an intron and that it is spliced *in vivo*. We modeled the mt-*rns* RNA secondary structure to evaluate the potential functional constraints of the intron insertion on the rRNA. In general group I or II introns occur at a particular position within the rRNA due to vertical inheritance of the allele containing an intron, homing or transposition (ectopic integration) in an intron-less allele/site, or due to horizontal transfer of the intron into a specific conserved target site [39]. Usually mobile introns insert into alleles that lack the intron by mechanisms that are site specific. Therefore, introns evolved strategies that optimize their dispersal, such as targeting highly conserved sequences present in every member of a population or even in other species. The distribution of introns in rRNA genes is not totally random, insertion sites appear to be found within conserved sequences usually near the tRNA and mRNA binding sites which span the interface between the small and the large subunits of the ribosome, suggesting a link between intron evolution and rRNA function [40]. Generally, domain III of the mt-*rns* RNA is located at the "head" of the small (30S) subunit of the ribosome to which ribosomal proteins such as S2, S3, S7, S9, S10, S13, S14 and S19 can bind [41].

The *O.ul*-mS952 intron is inserted in the lower half of the 16S rRNA domain III region which represents the interaction area with ribosomal protein S7 [42], [43]. Ribosomal protein S7 plays an important role in ribosome function as this protein is responsible for initiating the assembly of the 30S subunit. S7 is also one of the major ribosomal proteins to cross-link with tRNA molecules during the decoding process [44].

The presence of intron RNA in the mature rRNA could affect the ribosome assembly by altering the secondary structure or by blocking the tertiary or quaternary contacts and ribosomal protein interactions [45]. Unspliced or slowly splicing introns would prevent or interfere with ribosome subunit assembly and the formation of ribosomes which are deficient or not competent with regards to translational activity [46]. So given the location of the mS952 intron in domain III of the SSU any splicing deficiency could be detrimental or in some way affect the virulence of the strain.

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