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Research Papers

Molecular characterization of the rDNA-ITS sequence and a PCR diagnostic technique for *Pileolaria terebinthi*, the cause of pistachio rust

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Summary. Eleven samples of the most important pistachio rust (caused by *Pileolaria terebinthi* (DC.) Cast.,), which causes disease on Beneh (*Pistacia atlantica* Desf. subsp. *mutica* (Fisch. & Mey.) Rech. F) and Kasoor (*Pistacia khinjuk* Stocks.), were collected from herbarium specimens and pistachio fields at the Pistachio Research Institute in Rafsanjan, Iran. The complete sequences of ribosomal DNA internal transcribed spacers ITS1 and ITS2 (rDNA ITS) from the samples were determined and analysed. In general, very little rDNA ITS sequence variation was observed between rDNA ITS sequences of *P. terebinthi* samples. The length of the PCR fragments was 621 bp (for ITS1F-ITS4) and 1177 bp (for ITS1F-rust1), and consisted of 67 bp at the 3´ end of 18S rDNA, 93 bp of ITS1 region, 154 bp of 5.8S rDNA, 246 bp of the ITS2 region, 57 bp (for ITS1F-ITS4) and 613 bp (for ITS1F-rust1) at the 5´ end of the 28S rDNA. Restriction fragment length polymorphisms (RFLPs) of the rDNA-ITS region were used to identify *Pileolaria terebinthi*. Three strong bands of 105, 134 and 381 bp and five bands of 105, 134, 200, 301 and 437 bp are observed for the fragment of "ITS1F-ITS4" and "ITS1F-rust1", respectively. A PCR-RFLP diagnostic technique provided effective identification of the species by a unique pattern with the specific restriction enzyme XapI (ApoI).

Key words: *Pistacia*, ITS- rDNA, DNA Extraction, RFLP.

Introduction

Rust is one of the most important diseases of wild *Pistacia* spp., and is commonly known as pistachio rust on these hosts. The causal agents are *Pileolaria terebinthi* (DC.) Cast., 1842 (Cummins and Hiratsuka, 2003) and *Pileolaria pistaciae* Tai & Wei, 1933 (Bhardwaj and Sharma, 1994) that belong to the Pucciniales (formerly Uredinales) (Aime, 2006; Aime *et al.*, 2006). *Pileolaria terebinthi* is an autoecious macrocyclic fungus and one of the most important fungal pathogens of wild *Pistacia* species, especially *P. atlantica* subsp. *mutica* (Chitzanidis, 2002). *Pileolaria terebinthi* is known as the common rust fungus and has been reported in several areas including the western Mediterranean, mainland China and northwestern India. This rust has not been reported from native species (*Pistacia vera* L.) in North America (Chitzanidis, 2002). Pistachio rust was reported on pistachio trees of Gonbad in Iran by Petrak in 1956 (Petrak, 1956; Ershad, 2009). Inoculation of Beneh (*P. atlantica* subsp. *mutica*) and pistachio cultivars with aeciospores and urediniospores of *P. terebinthi* resulted in infection under greenhouse conditions (Hamzehzarghani and Banihashemi, 2002a).

Pistachio rust has potential to cause heavy economic losses in forest trees of Beneh and Kasoor (*Pistacia khinjuk* Stocks.) in Iran. These plants occupy a total area of approx. 2.5–3 million ha in several parts of the country (Padulosi *et al.*, 1996; Hamzehzarghani and Banihashemi, 2002b; Dehghani *et al.*, 2007). Losses up to 60% as a result of defoliation

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were reported from Egypt (Assawah, 1969) and 80% from blossom blight and die-back from Iran (Ali Dehghani, personal communication). The disease causes blossom blight, defoliation, branch deformity and die-back of affected plants. Leaves and petioles show round or irregularly shaped, reddish brown pustules. In early spring, these pustules occur on leaves and to a lesser extent on flowers, pedicels and fruits. Rust was first described on *Pistacia terebinthus* L. in 1815, and reported as *Uredo terebinthi* DC., (De Candolle, 1815); subsequently, the name was changed to *Uromyces terebinthi* (DC.) G. Win., (Saccardo, 1888), but this name was predated by *Pileolaria terebinthi* described in 1842 (Castagne, 1842). *Pileolaria terebinthi* has a complete life cycle, involving uredial (II) and telial (III) stages that have been observed in many countries on *Pistacia vera* (Petrak, 1956; Dinc and Turan, 1975; Corazza and Avanzato, 1985a; 1985b), *P. terebinthus* (Corazza and Avanzato, 1985b; Huseyn and Selcuk, 2004; Erdogdu *et al.*, 2010), *P. atlantica* subsp. *mutica* (Rabenhorst, 1871; Esfandiari, 1948; Ershad, 1995; Tavanaei *et al.*, 2004), *P. khinjuk* Stocks. (Dehghani *et al.*, 2007), *P. lentiscus* L., and *P. palaestina* Boiss. (Assawah, 1969; Bremer *et al.*, 1974; Chitzanidis, 1995; Bhardwaj, 1995, 1996; Guyot, 1951; Isikov, 1988). The spermogonial (0), aecial (I) and basidial (IV) stages were reported on *P. vera* leaves in Greece (Griggoriu, 1992; Chitzanidis, 1995) as well as on *P. atlantica* subsp. *mutica* in Iran (Hamzehzarghani and Banihashemi, 1999). The fungus overwinters as teliospores on fallen leaves. Teliospores can germinate and form basidiospores without a period of dormancy but they need alternating light and darkness for germination (Hamzehzarghani and Banihashemi, 2002b). The basidiospores are the infective propagules (Hamzehzarghani and Banihashemi, 2006), though little is known about the distance over which they can spread and cause new infections.

Analyses of nucleotide sequences of the ribosomal RNA gene to determine genetic variations, phylogenetic relationships and development of detection tools in rust fungi are widely used because of their multi-copy nature, interspecific diversity, and extensive availability in GenBank (Kropp *et al.*, 1997; Weber *et al.*, 2003; Abbasi *et al.*, 2005; Aime, 2006; Aime *et al.*, 2006; Chatasiri *et al.*, 2006; Barnes and Szabo, 2007; Alaei *et al.*, 2009). The rDNA unit is organized into three genes coding for the ribosomal units and two internal transcribed spacer (ITS) regions. To our knowledge, the present study is the first to focus on molecular characterization of *P. terebinthi* from Beneh. Specifically, the objectives of this study were to determine the complete rDNA ITS sequence of *P. terebinthi*, and to clarify and identify pistachio rust fungal species based on the PCR-RFLP analysis.

Materials and methods

Morphological identification

Fresh samples of pistachio rust were collected from *P. atlantica* subsp. *mutica* and *P. khinjuk* trees in pistachio fields of the Pistachio Research Institute in Rafsanjan, Iran (Table 1). Leaf samples were placed in plastic bags and processed in the laboratory within 2 days of collection. In addition, three pistachio rust samples (from *P. atlantica* subsp. *mutica*) were obtained as dried herbarium samples from University of Tehran, Karaj, Iran (Table 1). Although many attempts were made, no pistachio rusts could be found on commercial pistachio trees (*Pistacia vera* L.) in Kerman province. The presence of different stages (urediniospores and teliospores) was verified microscopically. Dried material was examined in lactophenol mounts (Maneval, 1936; Dhingra and Sinclair, 1995) by light microscopy (BH2, Olympus, Japan). Pertinent features were measured at ×200 or ×400 magnification, and at least 50 spores from each specimen were evaluated for spore measurements. Teliospores were checked for germination in a drop of sterile purified water on glass slides, after incubation in the dark in a miniature dew chamber at 15–20°C for 5 days (Hamzehzarghani and Banihashemi, 2002a).

Preparation of fungal material for DNA extraction

For dry herbarium and fresh samples containing *P. terebinthi*, spores were scraped from one rust sorus (telium) using a fine needle and suspended in 100 μL purified water in a microcentrifuge tube. The samples were kept at 4°C and allowed to soak for 24 h, after which the suspension with germinated teliospores was concentrated by centrifugation of 12,470 g for 5 min and removal of the supernatant. Spores were stored at 20°C until DNA extraction.

DNA extraction

The concentrated teliospores and urediniospores in each sample were manually ground in liquid ni-

Table 1. Details of rust samples and ITS cloned plasmid DNA used in this study.

Sample code[a]	Rust species	Host plant	Collection year, Province	Nature and source of sample
R219-1	*Pileolaria terebinthi*	*Pistacia atlantica* subsp. *mutica*	Unknown	Herbarium via M.Okhovat
R219-2	*Pileolaria terebinthi*	*P. atlantica* subsp. *mutica*	Unknown	Herbarium via M.Okhovat
R219-3	*Pileolaria terebinthi*	*P. atlantica* subsp. *mutica*	Unknown	Herbarium via M.Okhovat
IPR-1	*Pileolaria terebinthi*	*Pistacia khinjuk*	2006, Kerman	Field, Rafsanjan via A.H. Mohammadi
IPR-2	*Pileolaria terebinthi*	*P. khinjuk*	2008, Kerman	Field, Rafsanjan via A.H. Mohammadi
IPR-3	*Pileolaria terebinthi*	*P. khinjuk*	2010, Kerman	Field, Rafsanjan via A.H. Mohammadi
VRU-1	*Pileolaria terebinthi*	*P. khinjuk*	2008, Kerman	Field, Rafsanjan via H. Alaei
VRU-2	*Pileolaria terebinthi*	*P. khinjuk*	2009, Kerman	Field, Rafsanjan via H. Alaei
VRU-3	*Pileolaria terebinthi*	*P. khinjuk*	2010, Kerman	Field, Rafsanjan via H. Alaei
JAG-1	*Pileolaria terebinthi*	*P. khinjuk*	2011, Kerman	Field, Rafsanjan via P. Aminian
JAG-2	*Pileolaria terebinthi*	*Pistacia atlantica* subsp. *mutica*	2011, Kerman	Field, Rafsanjan via P. Aminian
R218	*Tranzschelia discolor*	*Prunus dulcis*	2002, Fars	Plasmid DNA via H. Alaei

[a] IPR,Iranian Pistachio Research Institute; VRU, Valiasr Rafsanjan University; JAG, Jihad-e-Agriculture.

trogen with a plastic pestle. After grinding, the DNA was extracted using a modification of the cetyltrimethylammonium bromide (CTAB) extraction procedure described by Alaei *et al.*, (2009). In brief, 400 μL extraction buffer (1.4 M NaCl (Sigma, St. Louis, MO, USA), 100 mM Tris-HCL (pH 8.0) (UCB), 0.02 M Na-EDTA (Invitrogen, Grand Island, NY, USA), 1% (vol:vol) β-mercaptoethanol (Sigma), and 2% CTAB (Sigma) were added to the pellet of the rust fungal spores after vortexing them briefly. To this buffer, 0.1 g zirconium beads (0.1 mm diameter) were added and the samples were exposed to two "bead-beating" cycles of 30 s each at the "high frequency" setting using a Mixer Mill MM301 (Retsch GmbH & Co.KG, Haan, Germany). The samples were briefly spun and then incubated at 65°C for 30 min. After 15 min treatment with 30 μg RNase A (Qiagen, Valencia, CA, USA), an equal volume of chloroform:isoamyl alcohol (24:1 vol:vol) was added to the sample. The mixture was emulsified using a vortex and subsequently centrifuged at 16,000 g for 5 min. The clear supernatant was transferred to a new tube and the nucleic acids were precipitated with isopropyl alcohol and centrifuged at 16,000 g for 5 min. The pellet was washed in 70% EtOH and recentrifuged. Finally, the pellets were dried at room temperature, resuspended in 50 μL of Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0],), and stored at -20°C. DNA concentrations were determined using the VersaFluor Fluorometer (Bio-Rad, Hercules, CA, USA) and the PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Invitrogen).

PCR amplification

PCR amplification of the rDNA ITS1-5.8S-ITS2 region was achieved with a GeneAmp PCR System C-1000 (Bio Rad, USA) thermocycler. The primer combinations were ITS1-F (5′-CTT-GGT-CAT-TTA-GAG-GAA-GTA-A-3′) (Gardes and Bruns, 1993) and Rust1 (5′-GCT-TAC-TGC-CTT-CCT-CAA-TC-3′) (Liu *et al.*, 1993) as well as ITS1F and ITS4(5′-TCC-TCC-GCT-TAT-TGA-TAT-GC-3′) (White *et al.*, 1990). The PCR reaction mixture (50 μL) contained GeneAmp PCR Gold Buffer II (10 mM Tris-HCl, 50 mM KCl; pH 9.0) (Applied Biosystems, Carlsbad, CA, USA), 2.5 mM $MgCl_2$ (Applied Biosystems), 0.2 mM of each dNTP (Roche, Mannheim, Germany), 0.2 μM of each primer, 1.25 units Ampli*Taq* Gold polymerase (Applied Biosystems), and 5 μL of diluted DNA extract. The DNA extract was usually diluted 1:10 and 1:100 before use. The PCR profile was: an initial

preheat at 94°C (5 min), 40 cycles of denaturation at 94°C (1 min), annealing at 44°C (1.15 min) for primer set ITS1F-rust1 and 45°C for primer set ITS1F-ITS4, and extension at 72°C (1 min), followed by a final extension at 72°C (10 min).

Cloning and sequencing of PCR products

The PCR products were gel-purified using 2.5% (w/v) NuSieve® 3:1 agarose gels (Lonza Bioscience, Basel, Switzerland) and the QIAquick Gel Extraction kit (Qiagen) to remove excess dNTPs and primers. PCR fragments were sequenced after cloning in plasmid vectors PCR2.1 TOPO® (Invitrogen). From the transformants, plasmid DNA was purified with the QIAprep® Miniprep kit (Qiagen) and checked for the expected insert size by *Eco*RI (Roche) and *Bst*Z1 (Promega) digestion and visualization by gel electrophoresis and EtBr staining. Three to five cloned inserts were sequenced by Macrogen (South Korea) in both directions using the SP6 and T7 primers. Three to five plasmid clones were sequenced. Sequences were analysed using Chromas 1.45 (1996–1998, Conor McCarty) and verified manually. All sequences were submitted to GenBank.

ITS sequences analysis

DNA sequences were compared to homologous sequences registered in GenBank using the standard nucleotide-nucleotide BLAST protocol (http://www.ncbi.nlm.nih.gov/BLAST/). In order to determine the extent and location of ITS sequence variation within and between the different rusts, the sequences were aligned using ClustalX 1.81 (Thompson *et al.*, 1997), followed by minor manual editing to uniformly present the location of nucleotides in repeat sequences.

PCR-RFLP analysis of the rDNA region

Because of the potential presence of *Tranzschelia discolor* (Fuckel) Tranz. and Lit., the causal agent of almond rust on trees in the same ecological niche of wild pistachio trees, a plasmid clone containing of the ITS regions of the ribosomal DNA of *T. discolor* prepared by Alaei *et al.*, (2009) was obtained and used in PCR-RFLP analyses. A part of the purified PCR products (7 µL) were digested overnight using 5 units of XapI (ApoI) (Fermentas, St. Leon-Rot, Germany) in a total volume of 20 µL. Each digest reaction consisted of 2.5 µL 10× buffer Tango™, 10 µL MiliQ water, 7 µL direct PCR product, and 0.5 µL restriction enzyme. The digestion mixture was incubated at 37°C water bath for overnight. All the digestion products were separated on a 2.5% NuSieve® 3.1 agarose (Lonza Bioscience, Switzerland) gel in TAE buffer, followed by staining with ethidium bromide and photographing under a UV trans-illuminator.

Results and discussion

Morphological identification

The pistachio rust samples from herbarium specimens and pistachio fields were morphologically identical. Microscopic examination of the pustules of the pistachio rust samples showed that they contained teliospores and urediniospores. Telia were mainly epiphyllous, dark-brown to black, irregular or roundish, merged, dusty, and 1–2 mm in diam. Teliospores were unicellular, globose-lenticular, reddish-brown and 35–37.5 × 30–37.5 µm in size; spore walls were chestnut brown and sparsely verruculose; the pedicels were persistent, 175–250 µm long, filiform and hyaline (Figure 1). Uredinia were mostly epiphyllous, reddish-brown, irregular or roundish, often on leaf veins and petioles, elongate, merged then naked, and 1.5–3 mm in diam. Urediniospores were brown, yellowish-brown, spherical, ellipsoid, pyriform mostly subglobose or oblong, 17–21 × 16–35 µm in size, with densely and minutely verruculose outer surfaces, 4–5 µm thick, and slightly thickened apically up to 6 µm, and each spore had four equatorial germ pores longer than aeciospores. Aeciospores were unicellular, globose to subglobose 13–22 × 18–34 µm, light brown in colour with dense verrucose walls. Spermatia were ellipsoidal, hyaline 3–7 × 4–5.5 µm and each formed at the end of a long spermatophore.

Amplification, cloning and sequencing

PCR amplification of the rDNA ITS region of *P. terebinthi* using the ITS1F-ITS4 and ITS1F-rust1 primer pairs was successful, and gave PCR products of 620 and 1177 bp, respectively. The sequencing of cloned fragments consistently produced good sequencing reads. All sequences were submitted to GenBank. Their accession codes are listed as HM639742–HM639746.

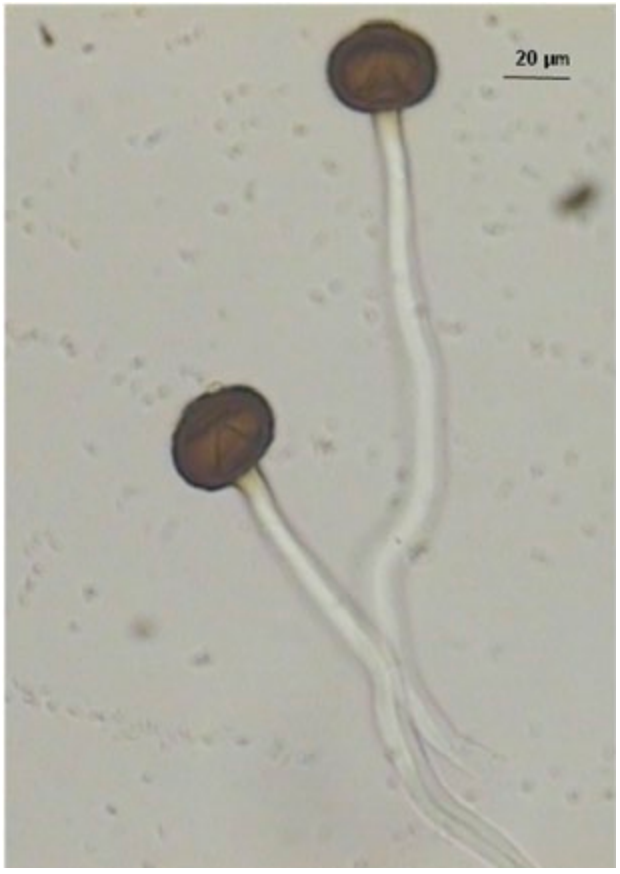

Figure 1. Teliospores (×200) of *Pileolaria terebinthi*.

Characterization of the rDNA ITS sequences

The complete genomic DNA sequences between the primers ITS1F-ITS4 and ITS1F-rust1 were obtained for pistachio rust fungal samples. The rDNA ITS nucleotide sequences of *P. terebinthi* showed high sequence homology. We did not observe any significant intraspecific variation. Further investigations into this feature, also using new markers such as Cox1 and D2-D1 region of 28S rDNA (LSU) are in progress. The GenBank-BLAST homology search using the *P. terebinthi* ITS sequences revealed *P. brevipes* Berk. & Ravenel as the most similar sequence (93% identity) with GenBank entries DQ323924 (rDNA-LSU), which is the partial sequence of 28S ribosomal RNA genes derived from *Toxicodendron* sp. collected from Clearwater, MN, USA (Scholler and Aime 2006). The length of the PCR fragments in our study was 621 bp (for ITS1F-ITS4) and 1177 bp (for ITS1F-rust1). This consisted of 67 bp of the 3´ end of 18S rDNA, 93 bp of ITS1 region, 154 bp of 5.8S rDNA, 246 bp of the ITS2 region, and 57 bp (for ITS1F-ITS4) and 613 bp (for ITS1F-rust1) of the 5´ end of the 28S rDNA (Figure 2). The nucleotide composition of the ITS1-5.8S-ITS2 sequences averaged 16.13% C, 36.49% T, 31.25% A and 16.13% G. A maximum of 8 bp changes were observed within the sequences of this rust fungus species. These changes consisted of a C to T change at positions 101, 234, and 358; A to G change at position 519; G to A change at position 811 and T to C change at position 1083.

PCR-RFLP analysis

When the PCR products from ITS1F-ITS4 and ITS1F-rust1 amplifications were digested with restriction enzyme Apo I, the results revealed length differences and species-specific restriction patterns for the ITS regions of *P. terebinthi*. The *P. terebinthi* digestion using enzyme XapI (ApoI) produced three strong bands of about 105, 134 and 381 bp but the *T. discolor* digestion produced two strong bands of about 380 and 329 bp (Figure 3A). The sequences of PCR product from ITS1F-rust1 amplification showed that XapI (ApoI) had four cutting sites to produce five bands, at 105, 134, 200, 301 and 437 bp, but the *T. discolor* digestion had three cutting sites and produced two bands of about 194 and 293 bp as well as two closed bands of 377 and 383 bp (Figure 3B). The PCR-RFLP diagnostic technique presented in this study provides an effective and sensitive method for indentification of *P. terebinthi*.

In conclusion, in this study we carefully determined the rDNA ITS sequence information from *P. terebinthi* the causal agent of pistachio rust. We also demonstrated that the PCR-RFLP method which can be used to rapidly and accurately identify *P. terebinthi*.

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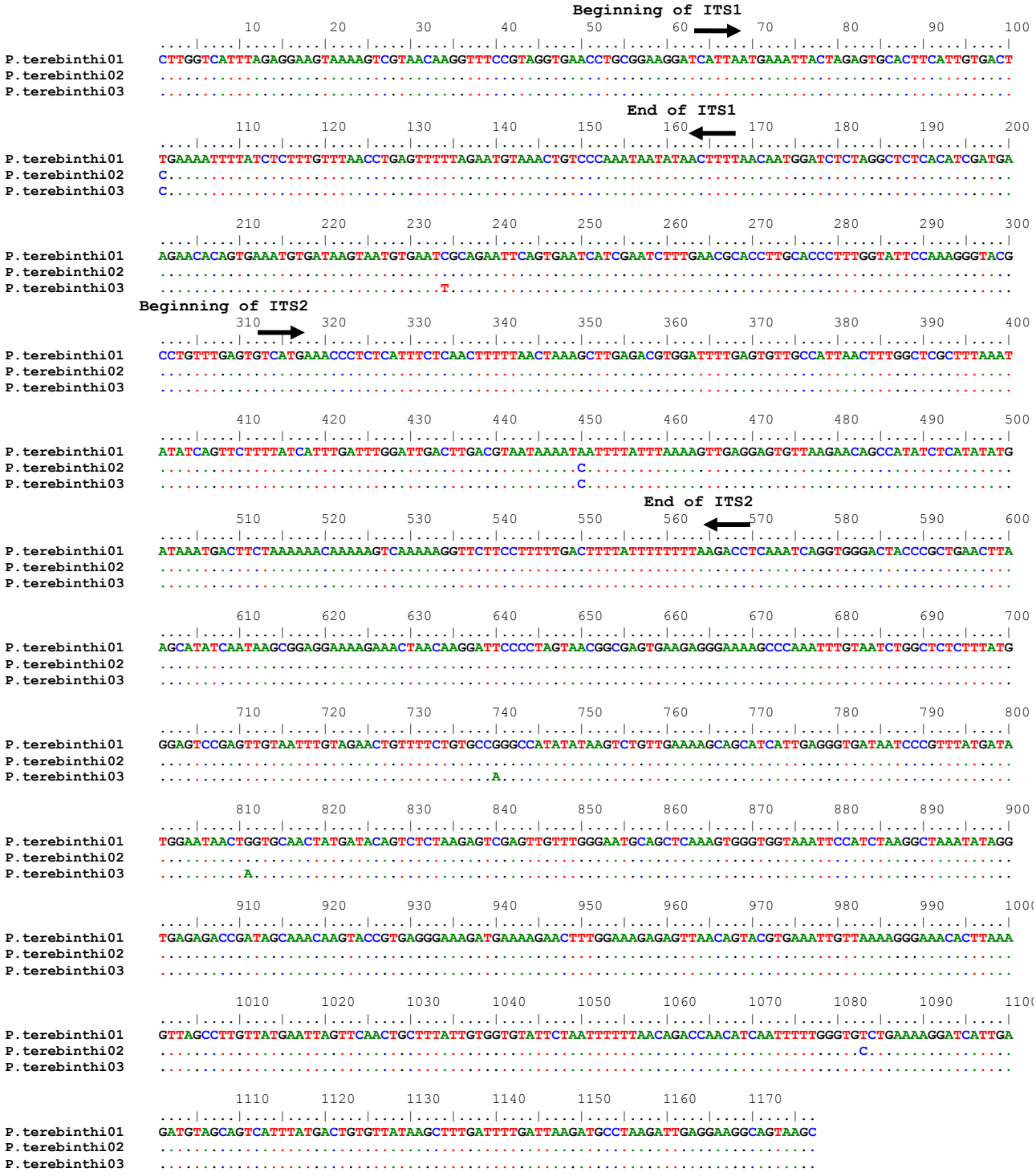

Figure 2. The complete ITS sequence alignment of *Pileolaria terebinthi* sample R291. The sequences are written 5′ to 3′. Identical nucleotides are indicated by dots. The ITS1 and ITS2 regions are marked with arrows. The sequence was conserved in all samples which differed at position 101, 234, 358 (T instead of C); at position 519 (G instead of A); at position 811 (A instead of G) and at position 1083 (C instead of T).

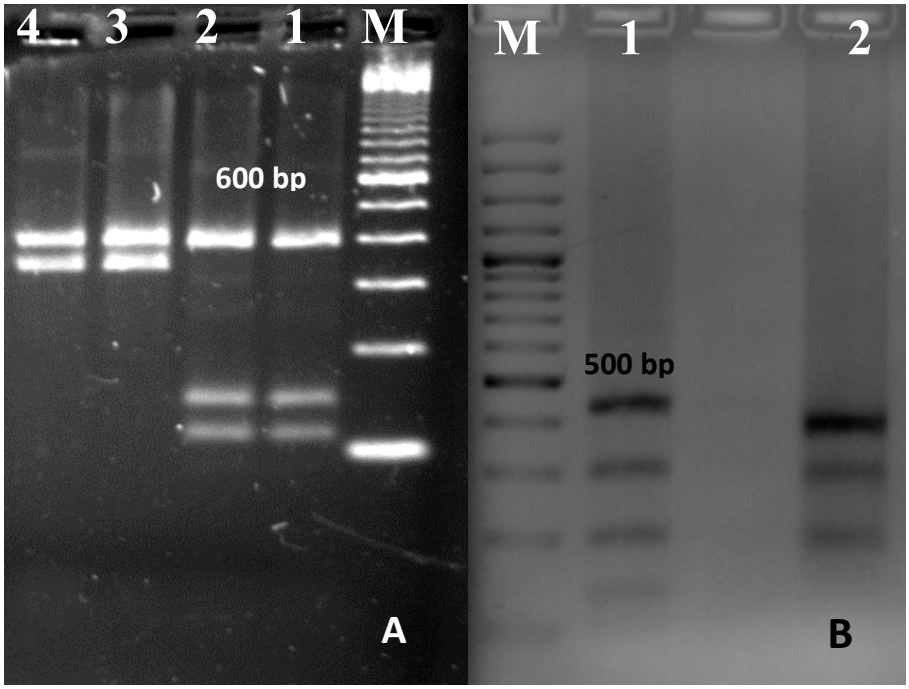

Figure 3. Agarose gel electrophoresis pattern of the amplified rDNA ITS region of pistachio and almond rust specimens digested with XapI (ApoI) using primer combination of ITS1F-ITS4 (A) and ITS1F-rust1 (B); Marker 100 bp (M), *Pileolaria terebinthi* Lane 1A (clone 02) and Lane 2A (clone 03), *Tranzschelia discolor* Lane 3A (clone 01) and Lane 4A (clone 03). *Pileolaria terebinthi* Lane 1B (clone 07), *Tranzschelia discolor* Lane 2B (clone 08).

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