# A Multigene Approach for the Taxonomic Determination of *Pleurotus eryngii* Isolates

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**Abstract:** The aim of the present work was to provide new data for the classification of two taxa of the *Pleurotus eryngii* complex, isolated from *Eryngium* spp. and *Ferula communis*, with molecular methods. Thus, we selected the multicopy loci strategy; the nuclear ribosomal internal transcribed spacers (ITS1, ITS2), the small subunit of the mitochondrial rDNA (mitSSU), the large subunit of the mitochondrial rDNA (mitLSU) and fragment from one single copy gene locus (the fifth and part of the sixth exon translation elongation factor) tef-1-α. The alignment of the sequences obtained showed no molecular variants able to distinguish the two populations in the ITS1 and ITS2 regions, in the small subunit of the mitochondrial rDNA (mitSSU) and large subunit of the mitochondrial rDNA (mitLSU). On the contrary, comparison of the tef-1-α sequences revealed the presence of four nucleotide substitutions between *P. eryngii* from *Eryngium* spp. and *P. eryngii* from *F. communis*, whereas no difference was found within each single population. These findings allowed us to identify significant differences at locus tef-1-α between *P. eryngii, Eryngium* spp. and *Ferula communis* such as to discriminate unequivocally the two taxa. In conclusion, such a multigene approach is more informative in comparison with a single gene approach for maximizing the chance to find possible molecular differences between two similar populations from the morphological and biochemical point of view.

**Key words:** Multigene approach, *Pleurotus eryngii*, internal transcribed spacers, taxonomic determination, *Nco* I, restriction site

## 1 Materials and Methods

### 1.1 Isolates examined and genomic DNA extraction

Sixty *Pleurotus eryngii* isolates gathered from several micologic areas of Sardinia were studied. Among these isolates, 30 were collected from *Eryngium* spp. and 30 from *F. communis*. The genomic DNA of all isolates was extracted from fruit bodies following the procedure reported by Marongiu et al.[1]

### 1.2 PCR amplification

DNA obtained from the isolates was amplified by PCR using primers specific for a region including the ITS1 and ITS2 loci, part of the smaller subunit of rDNA (mitSSU), part of the larger subunit of rDNA (mitLSU)[2] and a fragment (exon 5 and part of exon 6) of gene coding for elongation factor (EF-1-α).[3] The PCR reaction was performed in a 25 µL volume containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 11 mM $MgCl_2$, 0.1% gelatin, 200 nM dNTPs (Gibco-Brl), 1 unit of Taq polymerase (Red Taq DNA polymerase, Sigma), 0.625 ng of each primer and 20 ng of target DNA. The reaction was performed in a thermal cycler PCR-Express-Hybaid using different annealing temperatures according to each gene locus: 48°C for mitSSU, 53°C for mitLSU, 55°C for EF-1-α. The PCR consisted in a first denaturation at 94°C for 4 min, then 36 cycles of: denaturation (1 min.

at 94°C); annealing (1 min at the specific temperature); extension (1 min at 72°C) and a final extension step (10 min at 72°C). The PCR amplification of the region encompassing ITS1/ITS2 was carried out according to the protocol of Lee and Taylor.[2] The PCR products were checked by electrophoresis in TAE (Tris-acetate-EDTA) 1.5% agarose gel.[4] As reference standard, a molecular marker was used (1 kb DNA ladder, Invitrogen). All gel images were acquired and analysed with the Gel-Doc-2000 software (Bio-Rad) and showed all the products of expected length.

### 1.3 DNA purification and sequencing

The PCR products were purified through spin columns (Qiaquick PCR purification kit, Qiagen) following the manufacturer's instructions. For DNA sequencing the same primers as for the initial PCR reactions were selected. The nucleotide sequence of the PCR products was directly determined with a fluorescent Genetic Analyser ABI Prism 310 (Applied Biosystems) using the dye-terminators cycle-sequencing method. The reaction mixture included 0.5 µL of Cycle Sequencing Mix (DNA Sequencing BigDye Terminator kit, Applied Biosystems), 0. 32 pmol of oligoprimer, 1 µL of purified PCR product and sterile deionised $H_2O$ to 10 µL final volume. The following thermal profile was applied: 25 cycles of 10 sec at 96°C, 5 sec at 50°C, 2 min at 60°C using a thermal cycler GeneAmp 9700 (Applied Biosystems). At the end, the excess of dye terminator was eliminated by gel filtration (Centrisep Spin column, Princeton Separation, Adelphia NJ). The analysis of electrophoregrams was performed with the Sequencing Analysis software ver. 3.7 (Applied Biosystems) and the obtained sequences were compared with those in the NCBI database by using the Nucleotide Blast software (http:\\www.ncbi.nlm.nih.gov/blast/) BLASTN 2.2.6.[5] The nucleotide sequences obtained from the isolates and those of the NCBI database with the highest homology level were aligned with the CLUSTAL W (1.82) Multiple Sequence Alignments software.[6]

### 1.4 RFLP

Restriction reactions were carried out in 25 L volume containing: sterile deionized water, 10 L of EF-1 amplification product, 2.5 µL of 10X buffer and 5 U of *Nco* I enzyme. The mixture was incubated at 37°C for 2 hrs and then inactivated at 75°C for 5 min. Restriction fragments were analysed in 8% polyacrylamide gel stained with ethidium bromide.

## 2 Results and Discussion

The obtained sequences, except the sequence tef-1-α, were submitted to the NCBI database (Accession nos: AY639945; AY639944; AY639943; AY639942; AY639941; AY639940). Analysis of the sequences showed no difference between the two populations in the ITS1, ITS2, mtDNA SSU and mt DNA LSU regions whereas the comparison of the tef-1-α sequences between *P. eryngii* from *Eryngium* spp. and *P. eryngii* from *F. communis* evidenced four nucleotide substitutions (Figure 1); on the contrary, all sequences belonging to each population under study were identical. A restriction site for the enzyme *Nco* I was found in the EF-1α sequence of *P. eryngii* from *F. communis*, and it was not present in the sequence of *P. eryngii* from *Eryngium* spp (Figure 1). The digestion of *P. eryngii* from *F. communis* PCR product by *Nco* I produced two fragments of 439 and 145 bp, while the PCR product of *P. eyingii* from *Eryngium* spp. was not cut. Thus the significant differences in the tef-1-α locus between the two taxa provided an RFLP-based method for their unequivocal determination.[7] In conclusion, such a multigene approach proved to be more useful than the one-locus analysis method, as it increases the probability to find molecular differences between morphologically and genetically similar populations.





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A:    1   cgtgacttcatcaagaacatgatcactggcacctcccaggccgattgcgctatcctcatc  60
B:    1   cgtgacttcatcaagaacatgatcactggcacctcccaggccgattgcgctatcctcatc  60
A:   61   attgccgccggtactgtagaattcgaagctggtatctccaaggatggccagactcgtgaa  120
B:   61   attgccgccggtactgtagaattcgaagctggtatctccaaggatggccagactcgtgaa  120
A:  121   cacgctctccttgccttcactctcggtgtccgtcaactcatcgttgccatcaacaagatg  180
B:  121   cacgctctccttgccttcactctcggtgtccgtcaactcatcgttgccatcaacaagatg  180
A:  181   gacacaaccaaggtttgtgactcgcatttattagttgtgactaattttcctgagaatttt  240
B:  181   gacacaaccaaggtttgtgactcgcatttattagttgtgactaattttcctgagaatttt  240
A:  241   cgcagtggagcgaggaccgattcaacgaaatcatcaaggaaacctctaacttcatcaaga  300
B:  241   cgcagtggagcgaggaccgattcaacgaaatcatcaaggaaacctccaacttcatcaaga  300
A:  301   aggtcggctacaacccgaaggccgttgccttcgtccccatctcaggatggcacggtgaca  360
B:  301   aggtcggctacaaccctaaggccgttgccttcgtccccatctcaggatggcacggtgaca  360
A:  361   acatgttggaggagtccgtcaagtaagtacccatatgattgatctaaagaggcaatgatc  420
B:  361   acatgttggaggagtccgtcaagtaagtacccatatgattgatctaaagaggcaatgatc  420
A:  421   ttaccattttccagcatgacatggtacaagggctggaccaaggagaccaaggccggtgtc  480
B:  421   ttaccattttccagcatgccatggtacaagggctggaccaaggagaccaaggccggtgtc  480
                                  |
                                 NcoI
A:  481   gtcaagggcaagaccctcctcgatgccatcgatgccatcgaacccccgtccgccctcc  540
B:  481   gtcaagggcaagaccctcctcgatgccatcgatgccatcgaacccccgtccgccctcc  540
A:  541   gacaagcctctccgtcttcctctccaggacgtctacaagatcgg  584
B:  541   gacaagcctctccgtcttcctctccaggacgtctacaagatcgg  584
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**Figure 1. Alignment of *P. eryngii* from *Eryngium* spp. (A) and *P. eryngii* from *F. communis* (B) EF-1a sequences (584 nt single strand)**

A restriction site for *Nco* I is present only in *P. eryngii* from *F. communis* sequence (B); digestion produced two fragments of 439 bp and 145 bp.

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