A PCR based method to detect *Russula* spp. in soil samples and *Limodorum abortivum* roots in Mediterranean environments

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Abstract

**Aim of study:** Orchidaceae has the largest number of species of any family in the plant kingdom. This family is subject to a high risk of extinction in natural environments, such as natural parks and protected areas. Recent studies have shown the prevalence of many species of orchids to be linked to fungal soil diversity, due to their myco-heterotrophic behaviour. Plant communities determine fungal soil diversity, and both generate optimal conditions for orchid development.

**Area of study:** The work was carried out in the two most important natural parks in Alicante (Font Roja and Sierra Mariola), in South-eastern of Spain.

**Material and Methods:** We designed a molecular tool to monitor the presence of *Russula* spp. in soil and orchid roots, combined with phytosociological methods.

**Main results:** Using a PCR-based method, we detected the presence in the soil and *Limodorum abortivum* orchid roots of the mycorrhizal fungi *Russula* spp. The species with highest coverage was *Quercus rotundifolia* in areas where the orchid was present.

**Research highlights:** We present a useful tool based on PCR to detect the presence of *Russula* spp. in a natural environment. These results are consistent with those obtained in different studies that linked the presence of the mycorrhizal fungi *Russula* spp. in roots of the species *Limodorum* and the interaction between these fungal species and *Quercus ilex* trees in Mediterranean forest environments.

**Key words:** Detection; GIS; *Russula* spp.; *Limodorum abortivum*; PCR.

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This work has one Supplementary Figure and three Supplementary Tables.

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Introduction

Orchidaceae has the largest number of species of any family in the Plant Kingdom (ca. 20,000 species). There is evidence that many orchids are subject to a high risk of extinction due to natural or anthropogenic causes (Kindlmann et al., 2002, Nicol et al., 2005, Hutchings, 2010). Mediterranean forests are rich in endemic species, over 3% of the Valencian endemic plants are typical of forest ecosystems. *Limodorum abortivum* and *L. trabutianum* are very significant species in Mediterranean forests and it has been demonstrated that they depend on mycorrhizal fungi for seed germination and growth (Selosse et al., 2010, Selosse & Rousset, 2011, Tĕšitelová et al., 2012). *Limodorum* spp. present in the Font Roja Natural Park, also tend to associate with *Russula* spp. (Girlanda et al., 2006) in Italy and France Mediterranean forests. Molecular techniques have been applied successfully to the study of relationships between plants and fungi (Leake & Cameron, 2012, Sun & Guo, 2012). The main goal of this study is the development of molecular tools for detection of fungal presence in the Font Roja and Sierra Mariola Natural Parks (Alicante, SE Spain). This aims to create a useful tool for the study of presence and distribution of *Russula* spp., which is of vital im-
importance for the conservation of these Mediterranean orchids.

Materials and methods

Study site and sampling

The first study site was located in the Font Roja Natural Park, in the district of l’Alcoià (Alicante, SE Spain). The second site is the Sierra Mariola, a mountainous formation located between the provinces of Valencia and Alicante (Belda et al., 2009). Soil samples and small fragments of the roots of some L. abortivum individuals were taken from different sampling sites (Figure 1. and Suppl. Table S1 [pdf on line]). Each of the sampling points were defined by at least 10 random plots (200 m²) and vegetation was sampled according to the methodology, based on transects, called “quadrat technique” (Grant, 1981). The measure of total plant cover was also taken on the methodology of Braun-Blanquet (1965). Point samples were marked with a Trimble® GPS unit, using a spatial resolution of 1:5000. Georeferenced points were exported to ArcView® format (*.shp), to edit a localization map of the sampling points (Figure 1).

Isolation of total DNA from soil

For the isolation of total DNA from core soils, 5 g of core soil were suspended in 5 ml of T.E. 1X (10 mMTris-HCl, 1 mM EDTA; pH 7.5). This soil suspension was sonicated for 10 min at 4°C and centrifuged for 1 min at 2,500 rpm. Supernatants were recovered and this process was repeated twice, each time obtaining supernatants, which were pooled and centrifuged for 30 min at 11,000 rpm at 4°C. After centrifugation, the supernatant was discarded and we added 2 ml of CTAB-PVP lysis buffer (2% CTAB, 20 mM EDTA, 100 mMTris-HCl, 4 mM NaCl and 2% Polyvinylpyrrolidone) with 2 μl of 1:10 dilution protein K (Fluka) to the pellet obtained. The lysis mixture was incubated for 1 h at 65°C. After incubation, the samples were sequentially extracted with an equal volume of phenol, chloroform and isoamyl alcohol (1:1:24 V/V), centrifuged for 10 min at 14,000 rpm, the supernatant was extracted with an equal volume of chloroform, centrifuged for 10 min at 14,000 rpm, and precipitated with 2.5 V of 100% ethanol. The pellet was washed with 70% ethanol, air dried, and finally dissolved in 50 μl of nuclease free water.

Isolation of DNA from fungi and roots

CTAB-PVP based extraction method was used for the isolation of genomic DNA from 100 mg basidiospores of Russula spp. and from 500 mg orchid roots. Frozen tissues were crushed in a mortar, using liquid nitrogen and suspended in the lysis buffer and then incubated for 1 h at 65°C. Samples were subjected to phenol-chloroform extraction and isopropanol precipitation. The pellet obtained was washed with cold 70% ethanol, air dried and dissolved in 200 μl of TNE buffer (10 mMTris-HCl, 0.1 MNaCl, 1 mM EDTA, pH 7.5). Extracts were then treated with 1 μl of RNaseA (Sigma, www.sigmaaldrich.com) incubated at 37°C for 30 min, extracted with phenol-chloroform and finally precipitated with ethanol. The pellet was washed with cold 70% ethanol, air dried and finally dissolved in 50 μl of TE buffer. DNA from soil, roots and fungi was purified using a GeneClean spin kit (Q-Biogene Inc., Carlsbad, California).

Primer design for detecting Russula species

The primer design for specificity detection of Russula spp. was performed with PRISE (Fu et al., 2008). We used an ITS region of R. delica (Genbank acc. number: AF345250) as seed sequence. The hit table was generated using this seed sequence as a query in the BLAST server (http://www.ncbi.nlm.nih.gov/blast/), obtaining a 500 sequences for generation Hit table. The PRISE software generated 100 primer pairs, and we selected the primer pairs RusPrise1F-RusPrise1R (5’-
CACCCCTTTGTGCATCAC -3´ - 5´- CTTCATCGAT- 

of having a Tm greater than 55 °C, a length of 18 mer 

GCGAGAGC -3´) because it was satisfied the criteria 

naturation step at 94 °C for 5 min, followed by 35 cy-

www.promega.es). Reactions were started with a de-

at 72 °C for 5 min.

30 s and 94 °C for 30 s, and finally by an extension step 

42, 44, 47 and 50) for 20 s, and elongation at 72 °C for 

clones each with a gradient of annealing temperature (40, 

Macrogen sequence service (Macrogen Inc., Southern 

were sequenced by automated DNA sequencing at 

(Suppl. Table S2 [pdf on line]). The species with high-

plants and richness of species for each sampling point 

S1 and S2 [pdf on line]). We determined the dominant 

vegetative communities (Suppl. Table S1) was used to make an estimate of abundance and degree 

plant communities and Braun-Blanquet’s methodology 

be described above, except for the melting tempera-

Menetries, 1990). To verify that the bands obtained 

samples, except for those soils without presence in 

vegetation of Quercus ilex subsp. ballotula and L. abortivum 

The different protocols based on the CTAB Buffer 

used to obtain DNA from different tissues (roots and 

soil) allowed us to obtain sufficient quality DNA for subsequent PCR amplification. Fast 

and cheap protocols, used to obtain quality DNA for 

PCR, enabled us to process high volumes of diverse 

environmental samples.

A gradient PCR was carried out to verify the speci-

fity and annealing temperature of primers selected in 

silico. Figure 2A showed the amplifications patterns 

obtained from RusPrise primers. Using these primers 

we obtained a 158 bp band at the annealing temperature 

(Tm) of 55 °C, the Tm that was to detect the genus 

Russula spp. At this Tm no amplifications between 100 

and 200 bp, when a mixture of DNA from other fungi 

were found.

Detection of the presence of Russula genus in the 

different sampling points (Figure 1) is shown in 

Figures 2B and 2C. We obtained a single band for most 

samples, except for those soils without presence in 

the different areas (Suppl. Table S1 [pdf on line]). Thus, Font Roja 

has a climax community of holm-oak (Quercus ilex 

subsp. ballotula), although this is unusual in Mediterr-

anean environments (Richard et al., 2011).

We performed a gradient PCR using DNA from Rus-

sula basidiocarps, which was carried out using 5 µl of 

1:10 dilution of DNA, and a mixture of DNA as a control from soil fungus: Beauveria bassiana, Pochon-

nia chlamydosporia and Pochonia rubescens (50 ng 

each). PCR reactions contained, in a volume of 40 µl, 

the four dNTPs at 0.2 mM each (Fermentas, www. 

thermoscientificbio.com/fermentas/), two primers at 20 

pmol each, 2 mM MgCl₂, and 1 U of GoTaq (Promega, 

www.promega.es). Reactions were started with a de-

naturation step at 94 °C for 5 min, followed by 35 cy-

cles each with a gradient of annealing temperature (40, 

42, 44, 47 and 50) for 20 s, and elongation at 72 °C for 

30 s and 94 °C for 30 s, and finally by an extension step 

at 72 °C for 5 min.

Detection of Russula species in roots and soils.

To detect Russula spp. in roots from orchids and in 

different soils we used 1 µl of purified DNA from each 

sample with the RusPrise1F-RusPrise1R and ITS1F-

ITS4 primers. PCR conditions and product detection 

were described above, except for the melting tempera-

ture (Tm) which was fixed at 55 °C. PCR products from 

basidiocarps, Limodorum roots and soil from Font Roja 

were sequenced by automated DNA sequencing at 

Macrogen sequence service (Macrogen Inc., Southern 

Korea). Comparative sequence analyses were performed 

using the BLAST algorithm (http://blast.ncbi. 

nlm.nih.gov/Blast.cgi) and were deposited in the Gen-

Bank (JF415931-JF415933).

Results and discussion

We located L. abortivum populations and associated 

plant communities and Braun-Blanquet’s methodology 

was used to make an estimate of abundance and degree 

of coverage of vegetative communities (Suppl. Table 

S1 and S2 [pdf on line]). We determined the dominant 

plants and richness of species for each sampling point 

(Suppl. Table S2 [pdf on line]). The species with high-
est coverage was Quercus rotundifolia in areas where 

the orchid was present (RM1, RM2, FR1, FR2 and 

FR3). In contrast, Quercus cocciferae presented the 

major degree of coverage in areas where L. abortivum 

was absent (FRc and Mc). The dominant tree species 

was the only vegetation variable found in the different 

areas (Suppl. Table S1 [pdf on line]). Thus, Font Roja 

has a climax community of holm-oak (Quercus ilex 

subsp. ballotula), although this is unusual in Mediterr-

anean environments (Richard et al., 2011).

In this study we present a useful tool based on PCR 

to detect the presence of Russula spp. in a natural e-

vironment. These results are consistent with those 

obtained by the group of Girlanda et al., (2006), who 

linked the presence of R. delica in roots of the species 

Limodorum and the interaction between these fungal
species and *Quercus ilex* trees in Mediterranean forest environments.

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