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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 n 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 orchids 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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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, Těšitelová *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-

portance 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 m^2) 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 isoamylalcohol (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.

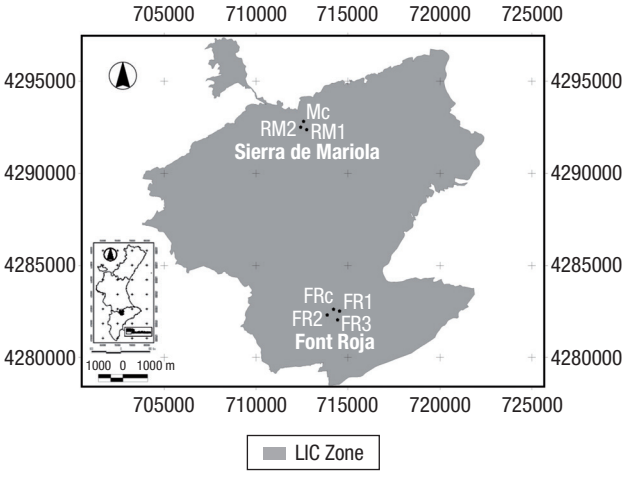

Figure 1. Study site and sampling points The sampling points from Font Roja (FR1, FR2, FR3 and FRc) and Sierra Marriola (RM1, RM2 and RMc) Natural Parks. Roots of the *Limodorum abortivum* orchid were collected from FR3, RM1 and RM2.

Isolation of DNA from fungi and roots

CTAB-PVP based extraction method was used for the isolation of genomic DNA from 100 mg basidiocarps 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´- CTTCATCGATGCGAGAGC -3´) because it was satisfied the criteria of having a Tm greater than 55 °C, a length of 18 mer and 100% sequence similarity with seeds sequence. The position of the selected primers in the ribosomal region is shown in Suppl. Figure S1 [pdf on line].

Optimization of RusPrise primers

We performed a gradient PCR using DNA from *Russula* 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, Pochonia 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 $MgCl_2$ and 1 U of GoTaq (Promega, www.promega.es). Reactions were started with a denaturation step at 94 °C for 5 min, followed by 35 cycles 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 temperature (Tm) which was fixed at 55 °C. PCR products from basidocarps, *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 GenBank (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 highest 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. *ballota*), although this is unusual in Mediterranean environments (Richard *et al*., 2011).

The different protocols based on the CTAB Buffer used to obtain DNA from different tissues (roots and basidiocarps) 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 specificity 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 vegetation of *Quercus ilex* subsp. *ballota* and *L. abortivum*. The band obtained was in a range between 100 and 200 bp, which matched with the expected band size according to the *in silico* predictions. As shown in Figure 2C, all samples were amplified using ITS1F-ITS4 primers, indicating that the absence of PCR inhibition. Note the large number of bands in the lanes corresponding to environmental samples, which are between 600-700 bp in size and match the size variations found in the ribosomal operon of most fungi (White *et al*., 1990). To verify that the bands obtained using RusPrise1F/1R primers belong to *Russula* spp., sequences from bands were subjected to analysis using BLAST algorithm (Suppl. Table S3 [pdf on line]). The sequences obtained from amplified samples from roots and the ground in the Font Roja had 100% identity with the sequence of different clones obtained in a previous study (Girlanda *et al*., 2006).

In this study 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 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

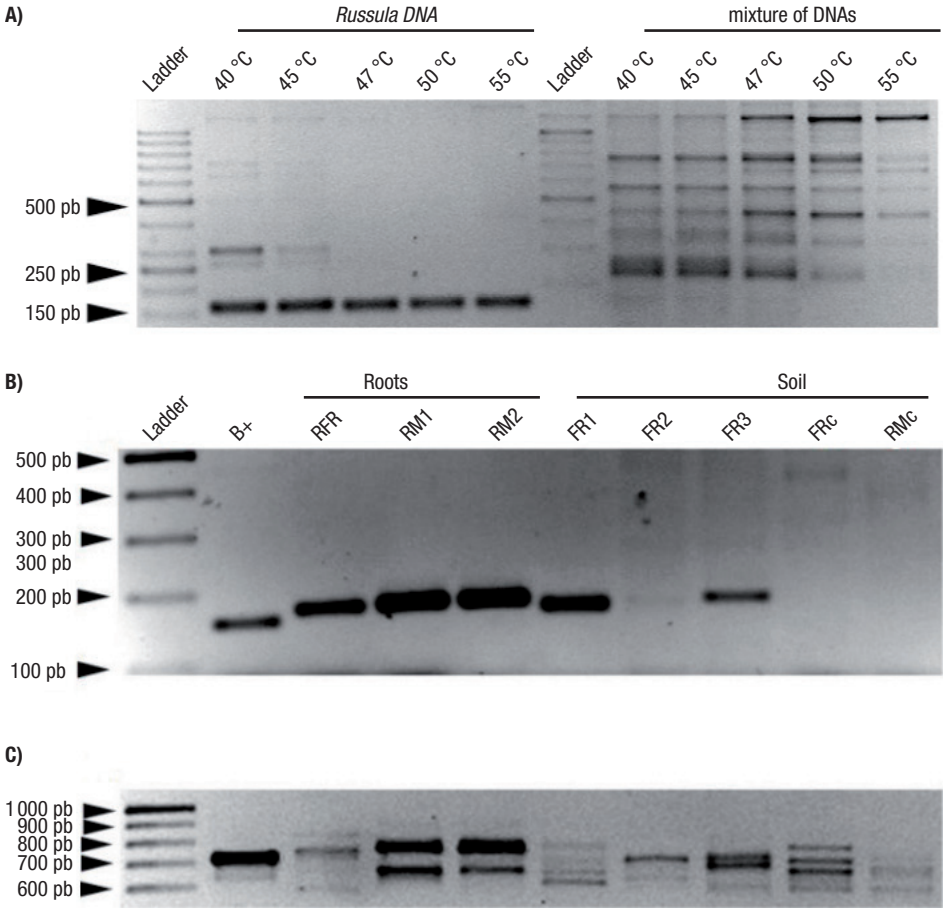

Figure 2. Optimization of primers RusPrise 1F/1R and detection of *Russula* genus in roots and soil. Panel (A) shows the PCR amplification obtained at different RusPrise 1F/1R primers and Tms. Gel lanes 2-6 correspond to PCR amplification from *Russula* DNA and gel lanes 8-12 correspond to PCR amplification from mixture of DNAs. Panel (B) and (C) show the amplification obtained from root and soil samples, using RusPrise 1F/1R and ITS1F-ITS4 primers. Lanes description: B+, DNA from *Russula* basidiocarp; RFR, roots of *Limodorum* from Font Roja; RM1-2, roots of *Limodorum* from Sierra Mariola; FR1-3, soil from Font Roja and FRc, MRc, control soils from Font Roja and Sierra Marriola.

species and *Quercus ilex* trees in Mediterranean forest environments.

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