Content Index

Figure index .................................................................................................................. 14
Table index .................................................................................................................. 16
Chapter 1. General introduction ..................................................................................... 19
  1.1. Oomycetes and the genus Phytophthora .............................................................. 21
  1.1.1. Water sources as hotspot for Phytophthora ...................................................... 24
  1.2. Species identification via DNA analysis: from DNA-barcoding to metabarcoding ........................................................................................................ 25
  1.3. Molecular methods for Phytophthora detection in environmental samples .......... 29
  1.4. From Sanger to Next Generation Sequencing technologies .................................. 32
Chapter 2. Objectives .................................................................................................. 47
Chapter 3. The use of genus-specific amplicon pyrosequencing to assess Phytophthora species diversity using eDNA from soil and water in northern Spain .......... 51
Chapter 4. Metabarcoding and development of new real-time specific assays reveal Phytophthora species diversity in holm oak forests in eastern Spain ....................... 83
Chapter 5. Environmental DNA sampling methods in aquatic environments suitable for Phytophthora metabarcoding studies ................................................................. 111
Chapter 6. General discussion .................................................................................... 141
Chapter 7. Conclusions .............................................................................................. 149
Index
Figure Index

Figure 1.1. Phylogenetic tree showing the relations between oomycetes, fungi and other kingdoms. Extracted from Rossman & Palm (2006) ........................................... 22

Figure 1.2. Sequence distribution of the most common DNA regions used for Phytophthora identification. Search was performed against NCBI Database, accessed on 25th January 2017 ................................................................. 27

Figure 1.3. Representation of ribosomal DNA (rDNA) polycistron from eukaryotic organisms, including the ITS1 and ITS2 regions, separated by the 5.8S gene and flanked by the 18S (small subunit, SSU) and 28S (large subunit, LSU) genes. Extracted and modified from Begerow et al. (2010) ........................................ 27

Figure 1.4. Sequence distribution of the DNA regions used for Phytophthora identification. Search was performed against Phytophthora-Database (Park et al., 2008), accessed on 26th January 2017 ................................................. 28

Figure 1.5. 5’ Nuclease Assay. During the annealing step, the primers and probe hybridize to the complementary DNA strand in a sequence-dependent manner. Because the probe is intact, the fluorescent reporter (D) and quencher (Q) are in close proximity and the quencher absorbs fluorescence emitted. In the extension step, the polymerase begins DNA synthesis, extending from the 3’ ends of the primers. When the polymerase reaches the probe, the exonuclease activity of the polymerase cleaves the hybridized probe. As a result of cleavage, the fluorescent dye is separated from the quencher and the quencher no longer absorbs the fluorescence emitted by the dye. This fluorescence is detected by the real-time PCR instrument. Meanwhile, the polymerase continues extension of the primers to finish synthesis of the DNA strand. Reproduced from PrimeTime®, Integrated DNA Technologies, 2016 ................................................................. 31

Figure 1.6. Historical development of next-generation sequencing technologies. The diameter of each bubble represents the sequencing read length of the platform [in base pairs (bp)]. Colors correspond to individual platforms. Extracted from Shokralla et al. (2012) .................................................................................................. 34 Index
Figure 3.1. Number of MOTUs generated using different score coverage threshold values based on ITS1 using a reference database of Phytophthora species ................. 63
Figure 3.2. Number of Phytophthora species detected and singletons produced per score coverage threshold in the control reaction ................................................................. 64
Figure 3.3. Unrooted phylogram based on nuclear ITS1 rDNA sequence analysis constructed with maximum likelihood approach .......................................................... 66

Figure 4.1. Detection limits of real-time PCR assay for Phytophthora quercina. Sensitivity was determined by testing a 10-fold dilution series of P. quercina DNA (isolate PS-982) ranging from 2 ng μL−1 to 0.2 fg μL−1 (a). Correlation coefficient (r²) is 0.998 (b) ................................................... 98
Figure 4.2. Detection limits of real-time PCR assay for Phytophthora taxon ballota. Serial dilutions of an environmental positive sample for P. taxon ballota (sample V1 confirmed by pyrosequencing) was used to determine assay sensitivity (a). Correlation coefficient (r²) is 0.983 (b) ................................................... 98

Figure 5.1. Proportion of Phytophthora species detected using isolation methods...... 123
Figure 5.2. Proportion of Phytophthora species detected by amplicon pyrosequencing. Dominant clade 6 species are represented with blue colors .......... 125
Figure 5.3. Multidimensional scaling analysis (MDS) showing similarity in community composition based on species relative abundance (Bray–Curtis distance). Three clusters were identified: group 1 (up left side) included all the samples from agricultural areas and most of the samples from forestry areas obtained from carnation baits; group 2 (down left side) included most of the samples from forestry areas obtained from Rhododendron bait and filters; and group 3 (up right side) included all bait and filter samples from two forest locations (PV1 and PV25) (Table 5.1) ........................................................................................................................................ 128
Table Index

Table 1.1. Sequence distribution of different taxonomic groups. Search was performed against NCBI Database, accessed on 25th January 2017 ........................................... 26

Table 3.1. BLAST results of the clustered sequences from the control reaction applying a barcoding threshold value of 99% ................................................................. 65

Table 3.2. Read distribution of species other from Phytophthora based on the ITS1 after BLASTn of the consensus sequences performed against GenBank. Reads from MOTUs with more than 99% similarity are in bold ........................................ 67

Table 4.1. Sequences of real-time PCR primers and double-quenched probes targeting the ITS1 rDNA region .................................................................................................. 91

Table 4.2. Phytophthora isolates obtained from culture collection and environmental samples used as positive and negative amplification controls in the real-time PCR assays. a Both P. quercina and P. taxon ballota were detected via pyrosequencing. b Only P. taxon ballota was detected via pyrosequencing. c Species other than P. quercina and P. taxon ballota were detected by pyrosequencing .............................. 92

Table 4.3. Read distribution per Phytophthora species in the 25 samples sequenced. BAL, P. taxon ballota; CAC, P. cactorum; GON, P. gonapodyides; NIC, P. nicotianae; PLU, P. plurivora; PSY, P. psychrophila; QUE, P. quercina; TEN, P. tentaculata; sp1–sp5, new phylotypes found. a FR, soil samples from Carrascar de la Font Roja; VS, soil samples from Vallivana; VSR, root samples from Vallivana. b Species detection using real-time specific PCR. +, positive sample; −, negative sample ................................. 94

Table 4.4. Frequency (%) of Phytophthora spp. detected and diversity and richness indices for the sampling areas studied. Least significant difference (LSD) test: means followed by the same letter do not differ significantly (P < 0.05). Lower case letters are for comparison of means in the same row, upper case letters are for comparison of means in the same column ................................................................. 100

Table 5.1. 454 reads generated in each of the sample included in the study, coordinates and main vegetation present in each of the river or stream ......................... 118