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Design and application of species-specific primers to *Quercus cerris* roots' identification in urban forests

Daniele Fantozzi, Gabriella Sferra✉, Dalila Trupiano & Gabriella Stefania Scippa

Accurate species identification, the first crucial step for effective root studies, is a time-demanding, experience-based and error-prone process. Molecular methods are therefore needed to ensure this process, especially in urban settings where root sampling is challenging. Here, we developed a novel molecular method for root identification in complex environments. Specifically, we focused on detecting *Quercus cerris*—a species common in European cities and non-urban areas and used in afforestation—from bulk root samples, including those collected non-invasively. To achieve this, we conducted the first comprehensive analysis of candidate DNA regions to discriminate among *Quercus* species. Among the candidate sequences tested, ITS and ITS2 showed the highest discriminatory power compared to commonly used barcodes such as matK, psbA-trnH, rbcL, rpoC1, trnL-trnF. Based on this results, we designed specific primers to target ITS and ITS2 and we developed a PCR-based protocol capable of reliability and specificity detecting *Q. cerris* within mixed *Quercus* root samples. This method was then successfully applied to root bulk samples collected via excavation and non-invasive soil coring in the urban area of Campobasso (central Italy), with results validated through traditional identification techniques. The outcome is a novel, rapid, low-cost, and non-invasive molecular approach for monitoring *Q. cerris* roots. More broadly, this tool enable in situ root identification and mapping which support the study of root functioning and dynamics in ecosystems and is particularly valuable in challenging urban environments.

Keywords Urban biodiversity, Root research, Root sampling, Soil coring, PCR-based methods, Barcoding. (Min.5–Max. 8)

Fine roots, with a diameter equal to or lower than 2 mm[1], are the primary organs the plants use to water and nutrient supply[2]. They are crucial to the plant adaptation to environmental conditions and, along with coarse roots, contribute to the "phenotypic plasticity" of the root system[3–6]. Their monitoring is a key to understand carbon flows, nutrient uptake and recycling and other processes related to the net primary productivity[7] and thus they are an optimal target to monitor the functioning and health of the entire root system[8,9].

In general, root studies are affected by the limited accessibility in the soil and by the less distinctive features of roots compared to above-ground parts[10–12]. Most studies rely on collecting roots from multiple individuals and species via soil coring[1,13,14]. These limitations are exacerbated in urban environments where roots must contend with limited space, physical obstacles and pavement barriers further complicating their study[15]. Additionally, root competition—defined as a reduction in the availability of a soil resource caused by other roots[16]—in urban settings assumes a further meaning: roots in resource-limited or resource-poor environments are commonly found in close proximity[11]. In fact, the scarcity of space and resources leads to highly dynamic, species-specific changes in the root growth, avoidance, and other competition strategies, which remain poorly understood[17]. Thus, the urban environment significantly affects the accuracy and precision of methods used to study rooting patterns and the rhizosphere, resulting in a major lack of information in the field[14].

A deep understanding of root biology in urban environments would be beneficial to the fundamental contribution the plants give as green infrastructures[14,18], helping to provide ecosystem services, promoting biodiversity and managing the plant- and root-mediated damages[19]. However, to pose species-specific questions or to address specific problems, the identification of taxa based on root samples represents a basic crucial step[12]. Beyond the general limitations of root studies in urban contexts, several challenges are also associated with species

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identification strategies. Some methods rely on functional traits that may vary under different environmental conditions and/or stressors (e.g. soil characteristics, growth conditions, stressors), while others—such as excavation, infrared spectroscopy—may be biased or impractical in urban contexts[11], or focus solely on above-ground plant parts[20]. Molecular-based methods, which rely on DNA differences among species, circumvent the environmentally induced variability associated with anatomical, morphological and other properties of the roots and bypass also the urban environment-induced problems for sample recovery and for the presence of roots from multiple taxa in the sample. Among molecular-based methods, DNA barcoding allows the identification of a specimen based on the specific sequence of a short DNA fragment that is the "barcode"[21]. This method is nowadays a standard procedure for sequence-based identification with a wide range of applications spanning from taxonomy and food fraud detection to biodiversity studies, including in urban environments[12,22,23]. To date, 534.736 plant specimens are recorded in BOLD database, a cloud-based data storage and analysis platform designed to support the generation and application of DNA barcoding[24]. Accurate species identification is essential for understanding biodiversity and for building reference libraries as a valuable comprehensive tool to be integrated with other data[25]. However, barcoding effectiveness relies on the availability of species-specific primers developed to differentiate targeted species from the others and that can way around the conventional drawbacks of the technique when dealing with complex plant material[26] like root samples[11].

Urban green areas are spatially diverse patches within the urban texture which include bushes, herbaceous plants, lawns and trees present in parks, public and private gardens, green roofs, residential yards, flowerbeds and traffic island beds[27]. These areas provide biodiversity-based ecological, environmental, and cultural benefits—comprehensively known as ecosystem services—central to human well-being and to urban sustainability[28]. Among all possible plant species included in urban green areas, trees—either individually or in urban forests—are the most relevant, as they are crucial in supporting biodiversity[29]. Recently *Quercus cerris* L. (Turkey oak), a deciduous tree native to southern Europe and Asia Minor and dominant in Mediterranean mixed forests[30,31], has gained attention for urban afforestation and reforestation projects. This is due to both its aesthetic and functional advantages, including tolerance to climatic extremes and drought[32,33]. Additionally, *Q. cerris* is included in a list of species for "urban and extra-urban afforestation plans"[34,35] which includes a variety of biological forms used in reforestation across Europe[36]. In Italy, this species is largely dominant in central and southern forests and, within the Molise region, has a wide distribution[37,38] also in urban contexts. The root system experiences more multiple stresses with respect to the above-ground parts of the plants and, in urban settings the stressors are exacerbated, especially in a climate change scenario[39]. The roots of *Q. cerris* are well-developed[40] and characterized by a complex multimodal seasonal dynamic with peaks in the mass and length of the fine roots occurring during the low-water content seasons[41]. However, also in the case of *Q. cerris*, more data and research on the roots are needed to link the main drivers of the root plasticity to the overall performance of the plant[1,3,42]. This would be beneficial not only to understand *Q. cerris* potential under the mutual influence among abiotic/biotic stressors and climate change, but also to drive the species management in urban environments. From a research perspective, *Q. cerris* is easily confused with *Quercus petraea* at a distance[43] and commonly grows in mixed stands with *Quercus robur*, *Quercus frainetto* and *Quercus pubescens*[32,38,44], which complicates species identification—especially when dealing with root material. Recently, sequencing technologies have opened new perspectives for studying *Q. cerris*[45,46], with promising applications in root research as well.

Assessing root distribution/presence allows a fine reconstruction of the rooting patterns and studying of the root traits in relation to the abiotic and biotic components[1,3,42]. Interpretations of any root parameter depend on precise identification of the below-ground organ, ideally down to the species or even individual level[47]. Species identification remains a critical challenge in root studies and usually it is achieved by a long experience-based, time-consuming and error-prone visual inspection. In this context, DNA barcoding, that implies the amplification and sequencing of a short fragment of a DNA to be diagnostic for species after comparison with a reference database, has great potential. In roots' research, species identification based on DNA barcodes is an assessed technique with a wide variety of applications ranging from distribution and diversity studies to medical plant authentication or monitoring also in relation to their interaction with human infrastructures[12,48–52]. However, its application in root studies is limited due to the lack of a universal plant DNA barcode, not yet achieved in plants, and by the complexity of the plant material from root samples leading difficult amplification and sequencing, especially if from urban contexts[11,14,17,26]. Root sampling, in fact, leads to having multiple plant species and multiple biological forms (e.g., trees, shrubs, herbs and grasses) within a single sample and DNA barcoding has been shown to be not suitable for the detection of multiple species from one sample[53].

In this scenario, our aim was to develop a PCR-based method for the molecular identification of the roots of *Q. cerris* from bulk roots sampled by soil coring or by excavation. To achieve this, we first performed a study that, based on the genetic divergence of seven typical plant barcodes (ITS, ITS2, matK, psbA-trnH, rbcL, rpoC1, trnL-trnF), attempted to identify the sequences of candidates which are good for discriminating among *Quercus* species. After, a procedure based on the candidate sequences was applied to design primer pairs which were subsequently used to amplify specifically the sequences of *Q. cerris*. Third, the PCR-based method was then applied to detect the target species in the DNA extracted from root samples allowing the identification of *Q. cerris* directly from bulk roots sampled by soil coring or excavation. Since the root samples were collected from urban contexts and from plants grown in controlled conditions, our approach offers a novel molecular tool to complement other techniques at the base of the monitoring of root biodiversity and interactions of *Q. cerris* also in urban contexts. The novelty of this study lies in the development of species-specific, PCR-based molecular methods for identifying *Q. cerris* roots directly from bulk root samples, including those collected non-invasively in complex environments such as urban ones. Unlike traditional approaches, our method was based on a comparative analysis of plant barcodes to select the most discriminatory and on the employment of custom-designed primers targeting *Q. cerris* specific regions (ITS and ITS2). Additionally, our method enabled the rapid, cost-effective and accurate detection of *Q. cerris* in mixed root samples providing a novel tool for

monitoring and assessing biodiversity in complex scenarios such as urban ecosystems. Our method would also support plant and forest management strategies, particularly under climate change conditions, by facilitating decisions on afforestation and reforestation involving *Q. cerris*.

Methods

The aim of our work was to develop a new method for the identification of the species *Q. cerris* to be widely and easily applicable in the field of plant research and specifically to root studies in complex environments such as urban ones. In this respect, we adopted a procedure (Supplementary Fig. 1) that allowed us: (i) to identify the most suitable marker for species-level resolution among *Quercus* species through sequence and genetic divergence analysis of the usual plastid and nuclear barcodes; (ii) to design and test primers specific to *Q. cerris* identification; (iii) to apply and validate the specificity of these primers in a PCR-based method to detect *Q. cerris* among other species from plant materials of diverse organs (leaf/roots) obtained by diverse sampling methods, including soil coring and excavation, also from complex environments such as urban ones to confirm the method's robustness and versatility.

Plant materials and DNA extraction

From February 2024, plant materials were collected within a garden area on the Apennines mountains in the region of Molise (Italy) located at ~ 860 m a.s.l. and within the area of the Municipality of Campobasso after the species identification by professional botanists and taxonomists present at the Department of Biosciences and Territory of the University of Molise (Prof. Gabriella Stefania Scippa and Prof. Paola Fortini, renowned botanist and taxonomist and, respectively, seed bank and herbarium curators). All plant materials were collected after receiving the related permits and no voucher specimen has been deposited in publicly available herbariums.

In detail, *Q. cerris* seeds were collected from the grounds and stored into a nylon bag. They were then set in soil and placed in a nursery until germination. The plants were irrigated twice a week during all months of their cultivation in the greenhouse, till their excavation in September 2024.

Fresh leaves of different *Quercus* species (*Quercus cerris*, *Quercus frainetto*, *Quercus ilex*, *Quercus petraea*, *Quercus pubescens*, *Quercus robur)* were collected in field and stored at -20 °C before DNA extraction.

Additionally, the roots of *Q. cerris* were non-lethally sampled from individuals within a specific site in the urban area of Campobasso (Molise, Italy), an area characterized by mixed stands of *Quercus* species[38] and one of the urban areas included in the studies of the National Biodiversity Future Center[54] aiming to monitor, preserve, restore and enhance the biodiversity of Italy. In detail, the Functional Urban Area of Campobasso was divided in 1 km^2 cells which were classified along a fragmentation gradient based on number and extent of green patches present[55,56]. Specifically, the sample site was identified as a urban forest of mixed stands of *Quercus* species. Sampling based on soil coring was executed for a segment of 10 cm with a diameter of 7 cm and the resulting material collected into a nylon bag stored at 4 °C. Then, soil cores were placed into a sieve with 2 mm of mesh size and washed with cold tap water and with distilled water till remaining only gravels and roots. These were air dried and hand-sorted to be processed for total DNA extraction. An analogous procedure was applied to extract the total DNA from the roots sampled from the excavation of *Q. cerris* plants grown in controlled conditions.

Root and leaf total DNA were extracted by a beads-based method by using DNeasy® Plant Pro Kit (Qiagen, Hilden, Germany) while total DNA from air-dried roots was extracted by DNeasy® PowerSoil® Pro Kit (Qiagen, Hilden, Germany) following manufacturer's protocol.

DNA quality/quantity was detected by spectrophotometer SmartSpec™ Plus spectrophotometer (Bio-Rad, Hercules, CA, USA) and isolated DNA was stored at -20 °C until further processing.

Sequence analysis

The ITS, ITS2, matK, psbA-trnH, rbcL, rpoC1, trnL-trnF were selected among the usual plastid and nuclear barcodes. All sequences were downloaded from the GenBank database of the National Center for Biotechnology Information[57], aligned and analyzed by MEGA software (version 11)[58]. Their genetic distance according to the Kimura two-parameters (K2P) model was assessed statistically by Wilcoxon signed rank tests executed in Rstudio[59]. A manual adjustment of the sequences and the removal of ambiguous regions was applied prior to the analysis.

Primer design and testing

The sequences of the candidates selected based on genetic divergence (ITS, ITS2) were submitted to Decipher package of R[60] to design suitable forward and reverse primers specific to *Q. cerris*. All designed primers were screened to meet some requirements about their length (16–25 bp), GC content (> 60%), presence of GC clamps, melting temperature (T_m between 50 and 72 °C), and selected only if meeting all of them. The forward and reverse primers resulting from this procedure were paired and each pair was retained for subsequent steps if the forward and reverse primer had a similar melting temperature (T_m), had no complementarity regions, and the PCR product was of a length higher than 150 bp. The resulting primer pairs were submitted to PrimerBLAST under standard settings[61] against GenBank to evaluate their ability of annealing with sequences other than the target species. Those with the higher specificity to the target species were considered eligible for being tested in the *in wet* procedures. Later, by an empirical inspection, a primer pair was selected for ITS (forward primer: CC CCACGCAGGCGGGAC; reverse primer: GCGCACGGGAGGCCAACT) and one for ITS2 (forward primer: C CGGTTCGGGCGGGGGC; reverse primer: CGTCGCCGAGGCAACGCGTAAG).

PCR optimization and amplification detection

Target sequences were amplified by PCR in the amplification mix composed by 12 µl of reaction mix, 0.125 µl of each primer (100 mM), 40 ng of template DNA and ddH_2O for a total volume of 25 µl. The amplification

	ITS	ITS2	matK	psbA-trnH	rbcL	rpoC1	trnL-trnF	Total
Quercus cerris	37	41	21	22	6	1	41	**169**
Quercus coccifera	48	54	77	74	63	1	26	**343**
Quercus frainetto	17	21	12	10	4	1	12	**77**
Quercus ilex	144	151	73	72	64	2	11	**517**
Quercus petraea	67	132	93	94	13	1	50	**450**
Quercus pubescens	47	53	22	14	8	1	72	**217**
Quercus robur	30	54	70	41	22	2	45	**264**
Quercus suber	90	95	15	13	7	1	61	**282**
Quercus trojana	15	19	7	7	2	1	11	**62**
Total	**495**	**620**	**390**	**347**	**189**	**11**	**329**	**2381**

Table 1. Summary of the sequence collected in genbank. Bold for total sequences available per each species or barcode and comprehensively.

Marker	ITS	ITS2	matK	psbA-trnH	rbcL	rpoC1	trnL-trnF
Sequence length (bp)	323–601	95–224	239–1515	262–458	310–1449	500–554	135–389
Alignment length (bp)	668	251	1515	503	1449	619	404
GC content (%)	51.4–66.9	50.3–70.7	29.3–36.9	21.6–29.4	41.9–45.5	42.8–43.7	25.9–31.4
Conserved sites	223	70	1480	375	1428	594	289
Variable sites	420	171	35	87	21	4	107
Informative sites	296	133	23	35	15	2	61

Table 2. Characteristics of the sequences of the candidate barcodes.

	ITS		ITS2		matK		psbA-trnH		rbcL		rpoC1		trnL-trnF	
Measurement	Mean	Dev.st	Mean	Dev.st	Mean	Dev.st	Mean	Dev.st	Mean	Dev.st	Mean	Dev.st	Mean	Dev.st
All inter-specific distance	0.050	0.028	0.062	0.042	0.009	0.007	0.018	0.014	0.002	0.002	0.002	0.002	0.013	0.014
Theta prime (θ′)	0.049	0.015	0.061	0.021	0.009	0.006	0.014	0.007	0.002	0.001	0.001	0.001	0.012	0.008
Minimum inter-specific distance	0.021	0.014	0.014	0.012	0.000	0.001	0.002	0.003	0.000	0.000	0.001	0.001	0.002	0.004
All intra-specific distance	0.024	0.028	0.031	0.042	0.003	0.005	0.009	0.013	0.001	0.002	0.003	0.001	0.005	0.013
Theta (θ)	0.024	0.008	0.032	0.018	0.002	0.003	0.008	0.005	0.001	0.001	0.003	0.001	0.005	0.007
Coalescent depth	0.115	0.112	0.169	0.209	0.007	0.008	0.036	0.032	0.005	0.004	0.003	0.001	0.033	0.036

Table 3. Genetic divergences of candidate barcodes.

was performed in a T100 termocycler (Bio-Rad, Hercules, CA, USA) with a pre-cycle step at 95 °C for 5 min, followed by 30 cycles made of an initial denaturation step at 95 °C for 30 s, the annealing step for 30 s at a temperature based on the T_m of the primers and the extension step at 72 °C for 30 s. These were followed by a further elongation step at 72 °C for 5 min. PCR products were checked by electrophoresis on a 1% agarose gel and by imaging using a GelDoc Go Gel Imaging System (Bio-Rad, Hercules, CA, USA). The gel image appearance was adjusted with Image Lab Software (Bio-Rad, Hercules, CA, USA).

Results

Analysis of sequences characteristics and of genetic divergence

In order to investigate the potential of the usual plastid and nuclear barcodes to discriminate among the species belonging to the genus of *Quercus*, we collected ITS, ITS2, matK, psbA-trnH, rbcL, rpoC1 and trnL-trnF sequences available in GenBank database (Table 1).

The sequences of each target were analyzed, and their characteristics reported in Table 2. The length of the target sequences was different with the wider range from 239 to 1515 bp for matK and the narrowest range from 95 to 224 bp for ITS2. Additionally, the sequences belonging to each target were only partially overlapping, as shown by the alignment length which is longer than the aligned sequences in the cases of ITS, ITS2, psbA-trnH, rpoC1 and trnL-trnF. Also, the guanine-cytosine content (GC content) was very variable showing psbA-trnH sequences with the lowest percentage, ranging from 21.6 to 29.4, and ITS2 sequences with the highest, with percentages ranging between 50.3 and 70.7. Furthermore, it was shown that ITS and ITS2 have the highest numbers of informative sites.

The genetic divergence in *Quercus* was evaluated by the estimation of six different parameters based on the K2P model, as reported in Table 3. The target sequences were characterized in their inter-specific divergence

through the average inter-specific distance, theta prime (θ′) and the minimum inter-specific distance, while the average intra-specific distance, theta (θ) and the average coalescent depth were used for the characterization of their intra-specific divergence. Additionally, the Wilcoxon signed rank test was used to confirm significant differences among genetic divergences. The targets showing the highest inter-specific diversity are ITS2 and ITS with, respectively, a mean equal to 0.062 and 0.050, while the lowest belong to matK (0.009).

The Wilcoxon signed ranked test confirmed significant differences among inter-specific variation for some of the target sequences in *Quercus* (Table S1) with a variation degree following the path ITS2 > ITS > psbA-trnH = trnL-trnF > matK > rbcL > rpoC1. Anyway, the ITS and ITS2 inter-specific variation significance was extremely greater respect all other target sequences.

At intra-specific level the diversity of ITS and ITS2 was greater than that of the other sequences (Table 3) and was equal to, respectively, 0.024 and 0.031. The Wilcoxon signed ranked test confirmed the significance of the differences among intra-specific variations for some of the target sequences (Table S2) with a variation degree following the path ITS2 > ITS > psbA-trnH = trnL-trnF > matK = rbcL.

As mentioned, the genetic distances were calculated both at inter- and intra-specific level for each candidate sequence and the related frequency distributions were analyzed in order to evaluate a possible barcoding gap. The barcoding gap is a sign for a sequence of being sufficiently diverging across species than within a species and supports its usage as an effective barcode for the species identification. The barcoding gap emerges if the frequency distributions of the inter- and intra-specific genetic distances are not overlapping. In other words, the more there is a difference between the greatest intra-specific distance and the smallest inter-specific distance, the more is present a clear barcoding gap. In our results, the distributions of the frequencies of inter- and intra-specific genetic distances were plotted for each barcode candidate (Fig. 1) to evaluate the possible presence of the barcoding gap. An obvious barcoding gap is not emerging for any of the sequence under analysis, although ITS, ITS2 and trnL-trnF showed less overlap among the distributions. Additionally, all targets but rpoC1 showed the intra-specific distribution mainly concentrated on the left side of the plot and the inter-specific shifted on

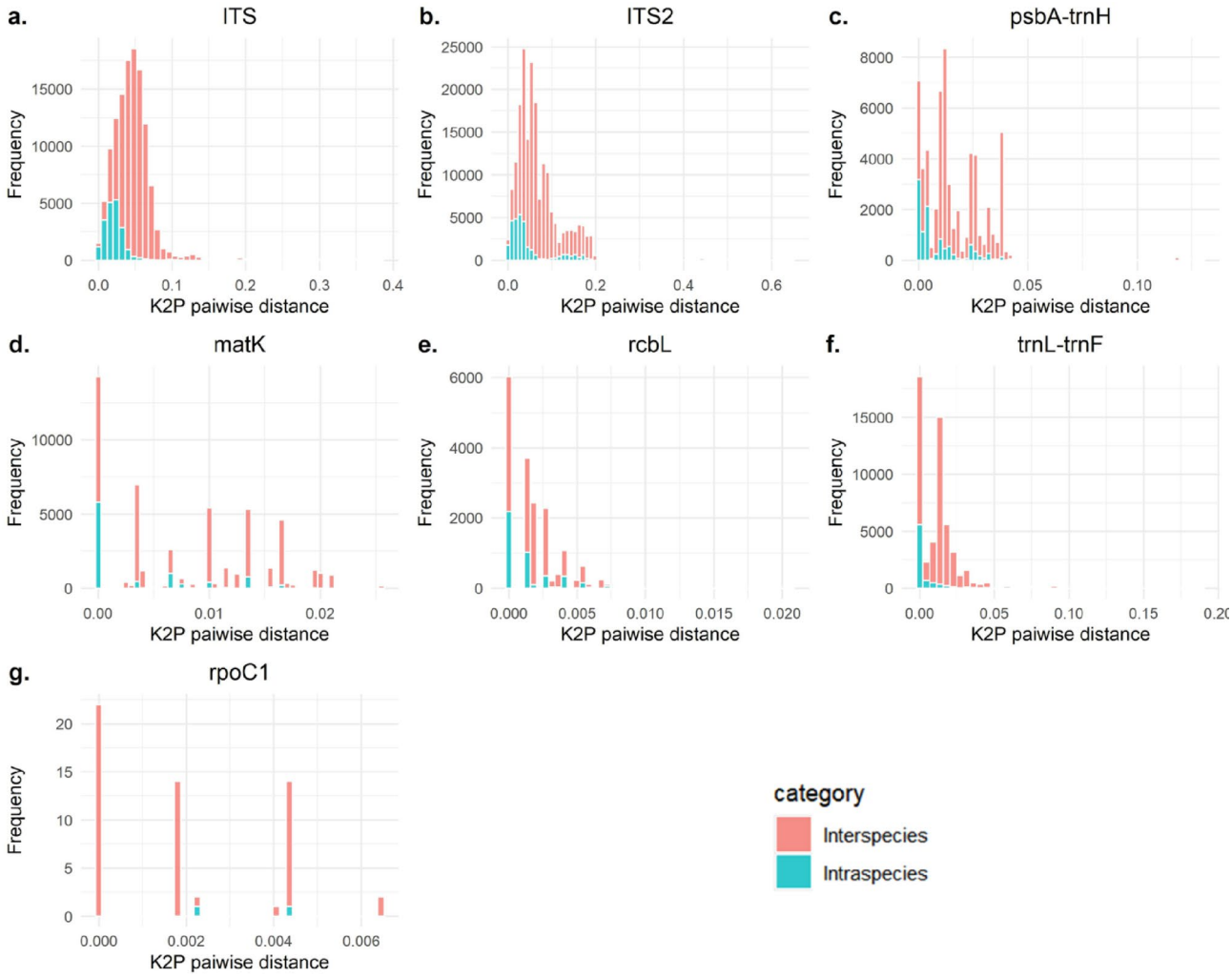

Fig. 1. Pairwise distance (K2P) distributions of sequence divergence for the candidate barcodes of *Quercus*. In red and blue respectively the distributions of the inter- and intra-species divergence of ITS (**a**), ITS2 (**b**), psbA-trnH (**c**), trnL-trnF (**d**), matK (**e**), rbcL (**f**) and rpoC1 (**g**).

the right-side. Ideally, the intra- and inter-specific distributions should be discrete and not overlapping for full barcoding effectiveness. However, our results indicate that the inter-specific variations are higher respect than the intra-specific variations which is anyway beneficial for an accurate identification of *Quercus* species.

Primer identification, in silico and *in wet* testing

Among target sequences, ITS and ITS2, showing the highest divergence, were used for the primer design process which flowchart is summarized in Fig. 2. All designed forward and reverse primers per each target sequence (ITS and ITS2) were screened to meet the requirements (see methods). This allowed the subsampling of 78 forward and 131 reverse primers for ITS and 75 forward and 244 reverse primers for ITS2 suitable for the in silico validation through primer-BLAST. Specifically, primer-BLAST testing was adopted to detect the primer specificity to the target species and allowed the selection of 23 primer pairs specific to ITS and 9 primer pairs specific to ITS2 of *Q. cerris* (Table S3).

All primer pairs were inspected according to their features and a pair for ITS and one for ITS2 were selected to be *in wet* tested. Before primer testing, *Q. cerris* DNA was extracted from fresh leaves as described in the methods and its concentration assessed by using the SmartSpec™ Plus spectrophotometer (Bio-Rad, Hercules, CA, USA). The primers designed on ITS sequence showed to be capable of amplifying efficiently their target from a template DNA quantity of 30 ng and allowed a PCR product of 361 bp as expected (Fig. 3). Also the primers specific to ITS2 were capable of amplifying the target from 30 ng of template DNA with a PCR product of 181 bp as expected (Fig. 3). Taken together, these results are also assessing the primers' sensitivity to the *Q. cerris* targets and serve as a guide for the setting of the optimal concentrations of template DNA to be used in the PCR reactions.

Additionally, to assess the species-specificity, the tested primer pairs were used in PCR reactions containing the DNA extracted from both target and non-target *Quercus* species as template. In details, six PCR reactions were set up based on a quantity of template equal to 30 ng of DNA extracted respectively from *Quercus cerris*, *Quercus ilex*, *Quercus pubescens*, *Quercus frainetto*, *Quercus petrea* and *Quercus robur*. The PCR conditions set as described in the methods allowed the specific amplification of the lonely ITS of *Q. cerris* and of the lonely ITS2 of *Q. cerris* (Fig. 4).

Efficiency of PCR amplification from urban-collected bulk root samples

Among the primer pairs used in the *in wet* procedures, those targeting ITS were selected for both their efficiency and species-specificity and used to test their ability to amplify the target directly from *Q. cerris* root samples collected by non-lethal methods based on coring and also by excavation from plants grown in the controlled

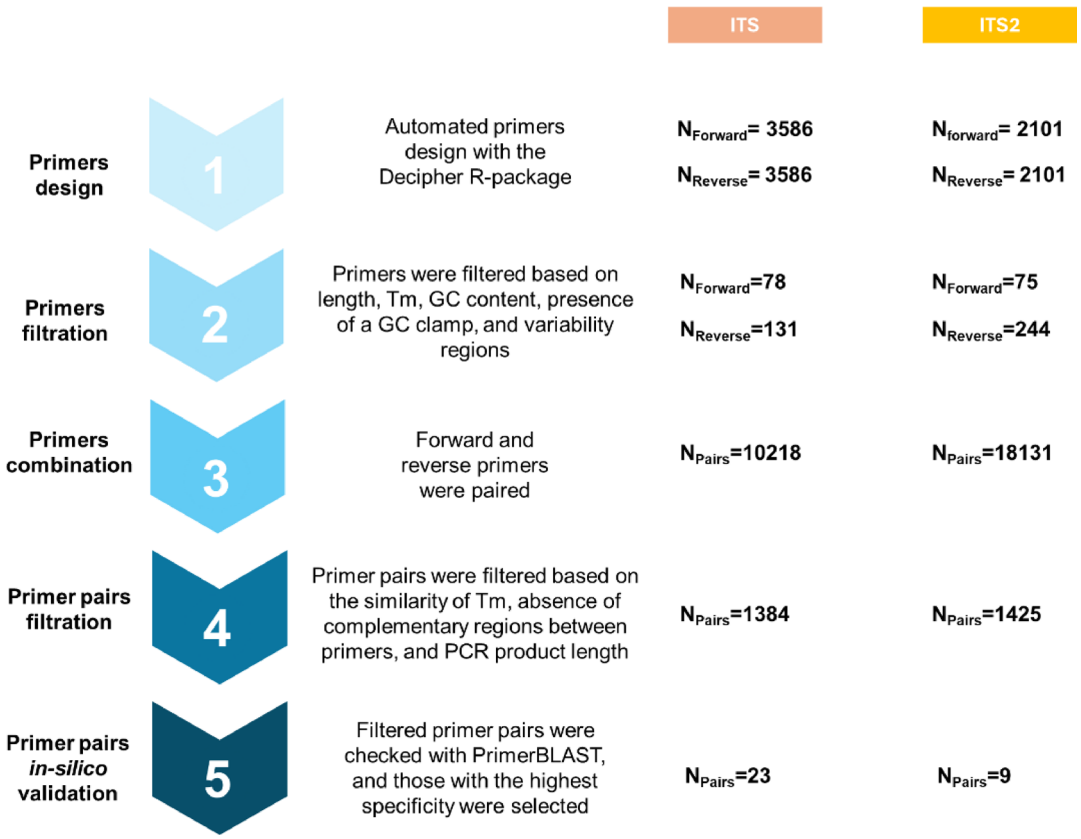

Fig. 2. Primer design process flowchart. $N_{Forward}$, $N_{Reverse}$, and N_{Pairs} indicating the number of forward, reverse primers or of primer pairs identified in each step (1–5) respectively for ITS or ITS2.

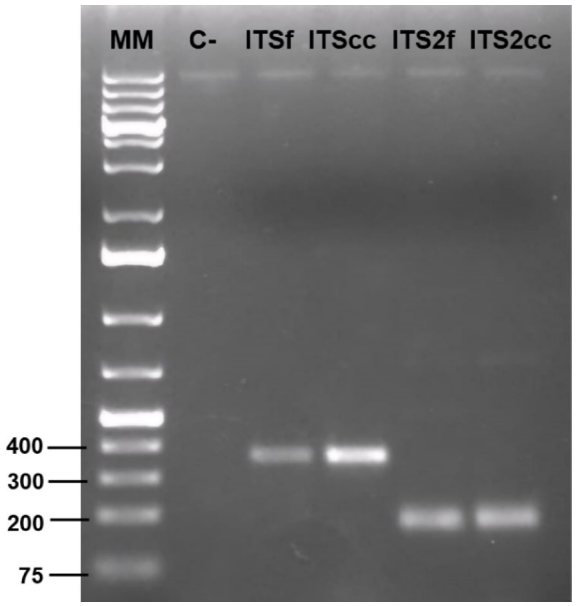

Fig. 3. PCR amplification results of the *in wet* primer testing on ITS and ITS2 from DNA template extracted from plant sampled in field (f) or grown in controlled conditions (cc). C- for negative control, MM for molecular marker. Original gels are presented in Supplementary Fig. 2.

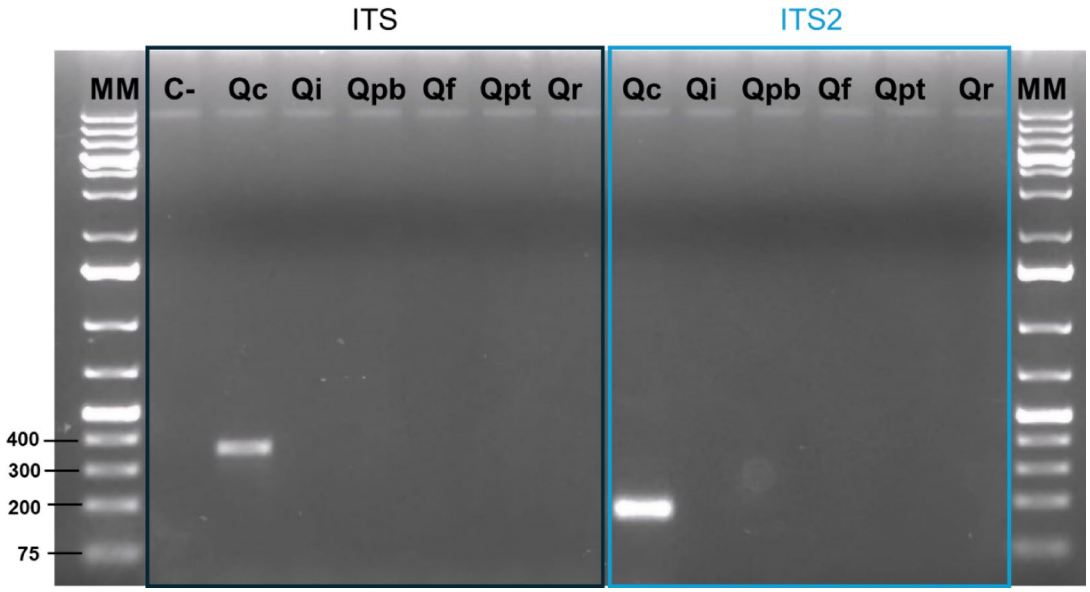

Fig. 4. PCR amplification results obtained using the primers for ITS (black box) and ITS2 (blue box) on target and non-target hit species: *Quercus cerris* (Qc), *Quercus ilex* (Qi), *Quercus pubescens* (Qpb), *Quercus frainetto* (Qf), *Quercus petraea* (Qpt) and *Quercus robur* (Qr). C- for negative control, MM for molecular marker. Original gels are presented in Supplementary Fig. 2.

conditions. The DNA was extracted in the first case directly from bulk root samples and, in the second case, from the roots sampled directly from the excavated plant grown in controlled conditions as described in the methods, reaching a concentration of 23.98 ng/µl and of 74.69 ng/µl, respectively. In both cases 30 ng of the extracted DNA deserved as template in PCR reactions under the conditions described in the methods that allowed an efficient amplification of the ITS sequence from both bulk root sample and excavated root sample (Fig. 5).

Discussion

Trees constitute an integral part of a sustainable urban environment and have increasingly been recognized for their contributions to climate regulation, human health, and habitat improvement[14]. Despite such benefits, trees

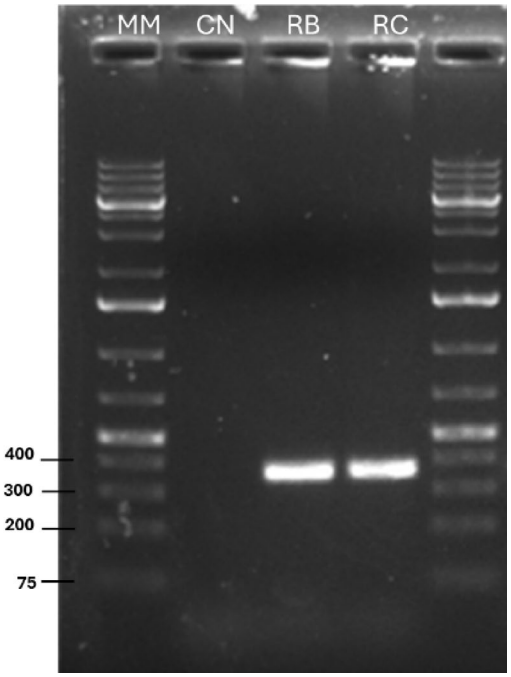

Fig. 5. PCR amplification results based on the DNA extracted from bulk root samples (RB) and from excavated root samples of a plant grown in controlled condition (RC). CN for negative control, MM for molecular marker. Original gel present in Supplementary Fig. 3.

cause certain ecosystem disservices insidiously endangering public properties and structurally compromising sidewalk surfaces, and underground pipes[62]. Multiplicity of causes has been connected to tree species[63], soil-space limitations/composition[64–66] and the proximity of planting holes to sidewalks[67]. Generally, the roots of fast-growing species such as poplar (*Populus* L.), eucalyptus (*Eucalyptus* spp.), and ficus (*Ficus microcarpa Linn. f.*) tend to induce damage[14]. However, roots of different species may occupy different realized niches. When several species are growing in mixtures or limited portions, it is expected to explore a broader space (i.e. soil volume) and produce more biomass than each of the species separately[68–70]. Nonetheless, there are still knowledge gaps in understanding tree root damage due to a lack of methodologies underpinning proper and effective management/monitoring of plants in urban patches[1,71]. Additionally, in urban contexts, the presence of obstacles and other physical impediments further limits the access to the roots and their sampling and monitoring need novel non-disruptive methods[14]. Thus, root studies, which have the potential to reveal the links among the organ functional traits and the whole plant health and stability, are biased due to hidden nature of the organ and complicated by the hard species identification based on root morphology[1,12].

In this scenario, we aimed to develop a novel molecular tool based on a non-disruptive protocol to identify and monitor the roots of *Q. cerris*, a species for afforestation and reforestation in urban context[34–36,72] and of economic interest[73] – from bulk roots samples. This method was also tested on excavated roots. Thus, our work can be divided into three steps. First, we performed a study that, based on the genetic divergence of seven typical plant barcodes (ITS, ITS2, matK, psbA-trnH, rbcL, rpoC1, trnL-trnF), attempted to identify those best suited for primer development for species identification among *Quercus* spp. Second, we designed species-specific primers. In detail, after selecting barcode candidates, we designed primers, and validated them both in silico and *in wet*. Third, we used the validated primer pairs to develop a PCR-based approach applied to detect the presence of the target species in DNA extracted from bulk roots sampled via soil coring or excavation, in both urban environments and controlled conditions.

About the first step, we analyzed the DNA regions of seven candidate barcodes of *Quercus* species collecting relevant data about their sequences from databases to overview their genetic divergence. Ideally, a DNA barcode must have sequence variation, conserved flanking loci and short target DNA[74]. Among candidates, we found ITS and ITS2 to have more characteristics fitting with those of a good barcode: high number of informative sites, high number of variable sites contemporary to a comprehensive short sequence length. Additionally, our results showed also the GC content of ITS and ITS2 to be higher than all of the other candidates, which is beneficial to primer clamping and specificity. The sequences of ITS and ITS2 were already included in studies on *Quercus* species to decipher their phylogenetic relationships and evolutionary distance[75,76]. These sequences have been targeted also by other polymorphisms-based methods to characterize the phylogeny of Mediterranean evergreen oaks[77] and were included in a study pointing out a latitude-based separation of the oak subgenus *Cerris*[78]. So far, ITS and ITS2 have been used for the identification of thousands of plant species[26] and as nuclear markers are characterized by a higher evolutionary rate respect the chloroplast ones[79] which is beneficial to taxa identification. In fact, despite the finding of plant species-specific regions is difficult, various authors attempted

to design primer specific to ITS sequences also for economically relevant species[80–82]. Our findings, therefore, support the use of ITS and ITS2 as effective barcodes for distinguishing *Q. cerris* from other species.

The results showed, also, that ITS and ITS2 have the highest inter-specific genetic distance and the lower intra-specific genetic distance among the sequences of the *Quercus* genus included in the study. We do not detect the minimum inter-specific distance to be higher than the coalescent depth as previously described for other genera[83]. In fact, we observed a partial overlap among inter- and intra-specific genetic distance distributions that is expected while including closely related taxa in the analysis[84]. Additionally, ITS and ITS2 of *Quercus* are known to undergo a rapid evolution rate and accumulate both intra- and inter-specific mutations which may conceal the inter-specific genetic divergence among *Quercus* species[85] leading to a smaller difference among inter- and intra-species divergence. Despite of this, ITS has been tested for the species discriminatory power on Chinese oaks and allowed the highest species resolution and thus is recommended as core barcodes for *Quercus*[85]. Also ITS2 demonstrated to increase the species resolution during a study on the Euro-Mediterranean oaks[86].

In the second step of our work, we used ITS and ITS2 as candidate barcodes to design and test primers, both in silico and *in wet*. We designed multiple forward and reverse primers, paired them, and evaluated their specificity to the target species using in silico methods. Specifically, we tested each primer pair via BLAST searches in GenBank to assess their specificity to *Q. cerris*. As noted earlier, *Quercus* ITS and ITS2 regions evolve rapidly and accumulate both intra- and inter-specific mutations[85]. The BLAST results indicated high specificity for the target species but also showed some potential cross-reactivity with other *Quercus* species. It has to be noted that the blasting was performed under default settings of Primer-BLAST which, in terms of "primer specificity stringency", adopt a large cut-off (5 or less mismatches) to include a species in the possible target list. In this regard, we have adopted severe criteria to select primers specific to the target taxon. In particular, we retained the primers annealing in the region of higher variability. This stringent selection allowed the identification of primer pairs to be submitted to the *in wet* testing. The results that we obtained by the *in wet* primer testing, in fact, confirmed the sensitivity and specificity of the amplification of ITS and ITS2 of *Q. cerris*, since low quantities of template DNA per reaction were used and none of the non-target species was amplified. In this perspective we advise the PCR assay conditions needed for *Q. cerris* detection.

In the third step of our work, we applied the PCR assay to detect *Q. cerris* in bulk root samples under the presence of multiple species. DNA was extracted from the roots collected via soil coring or excavation and used as a template for PCR with our ITS- and ITS2-specific primers. This procedure allowed us to specifically amplify the target sequences of *Q. cerris*. Our method demonstrates potential as an accurate, efficient and inexpensive PCR-based strategy to identify tree roots in afforestation and reforestation programs. It provides a valuable approach for ecological studies of *Q. cerris* roots and can enhance tree management strategies. Root samples, typically, are characterized by the presence of multiple-taxa and kingdoms based on tight interactions occurring with the roots of other species[53], with the microbial community present in the rhizosphere and with other biotic factors such as pests and pathogens[87]. Due to this, a single organism is hard to physically isolate from associated interactors, and this may lead to false positive results when applying DNA barcoding approaches to plants[53,88]. Metabarcoding has the potential to overcome this, but it is still expensive, time consuming, and complex compared to conventional PCR and sequencing, especially in the case of a single species as target[88]. Additionally, in plants standard barcodes do not provide species level resolution, and their usage must be tuned based on genetic divergence and taxonomical complexity of the group of the target species[11,14,17,26,89]. Thus, the identification by PCR of a single or multiple species in complex mixed samples remains the most effective, cheap, fast and feasible method to achieve the goal and would be beneficial especially while working with multi-species root samples from urban contexts[90,91].

The ideal technique to identify species from root samples would be not only time and cost effective, but also accurate in the assignment of multiple species and possibly quantitative. Thus, our PCR-based identification of *Q. cerris* can be implemented to identify multiple species, but the higher the number of targets, the higher the number of regions requiring a successful amplification[92]. Therefore, future studies developing multiplex PCR require a careful dissection of the genetic divergence among target sequences[93]. Similar to how sequence-based studies benefit from databases like BOLD or GenBank, PCR-based analysis would also benefit from a reference database containing detailed information on reaction characteristics, expected amplicon size, and species-specific outcomes. This would help improve assignment accuracy and reduce false positives – especially in species-rich samples[11,92]. Although our method currently provides qualitative detection, quantifying root DNA could yield insights into root functions and responses[94,95]. Therefore, our method opens new avenues for developing multiplex and qPCR tools to study *Q. cerris* roots and their functioning in situ.

Conclusions

Overall, our study relates to specific outcomes of interest, identifying the best targets to be used for discriminating among *Quercus* species based on barcode genetic divergence and defining a PCR-based protocol to successfully and specifically amplify *Q. cerris* also in complex samples. Root research will particularly benefit from applying this molecular technique because no methodology exists today to monitor the entire root system architecture of large-sized organisms such as *Q. cerris*. It will be also a crucial procedure mapping the horizontal/vertical distribution of the roots and, thus, understanding their ecosystem functioning both in natural and urban contexts. The knowledge about roots can be used to develop strategies to greening establishment and maintenance and may drive the operational plans about "where" and "how" use different species according to the urban texture. This is particularly relevant in urban contexts where plants play the crucial role of being essential for city sustainability and health but also possible threat to infrastructure and residents. About 50% of tree decline across European countries occurs because of a failure of the interplay between the above- and the below-ground tissues and these events are fated to increase due to climate change impacting on a higher frequency and intensity of

storms and other highly impacting climate events. Thus, new rapid non-disruptive methods – as our PCR-based strategy—are needed for a precise species assignment allowing correct root monitoring and mapping especially for tree species. It will be also decisive in proper maintenance and management of plants, and trees specifically, and in maximizing/optimizing their capacity in delivering ecosystem services and actions as nature-based solutions, functional in both non-urban and urban environments. Specifically, it could contribute to support urban greening needs with appropriate environmental policies based on tight interactions among policy actors and researchers which must be guided by a better understanding of the urban ecosystems and of their value in the scenario forecast of urban changes. Indeed, according to the European Commission, as described in its EU Biodiversity Strategy 2030, more than 3 billion trees are expected to be planted within 2030 to enhance tree canopy in urban areas. This policy document, calling all the European municipalities to develop greening plans and projects, needs to be integrated by rules and guidelines about how to establish and monitor the trees for their maintenance and effective long-term benefits.

However, further research is needed to move our PCR method to quantitative ones, which would drive a proper quantification of belowground root presence of the target species, or to multiplex, allowing the simultaneous identification of multiple species. We hope that this information can represent a molecular tool useful as an indicator of *Q. cerris* presence also in the soil and that the knowledge that we obtained is valuable to drive further studies also on the development of molecular methods targeting other *Quercus* species.

Data availability

All data generated or analysed during this study are included in this published article and/or available under request to the corresponding author Gabriella Sferra (gabriella.sferra@unimol.it).

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D.F.: conceptualization, data curation, formal analysis, investigation, methodology, software, validation, visualization, writing—original draft; G.S.: conceptualization, data curation, formal analysis, investigation, methodology, software, supervision, validation, writing – original draft, writing—review & editing; D.T.: conceptualization, project administration, supervision, writing—review & editing; G.S.S.: funding acquisition, project administration, resources, writing—review & editing.

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Declarations

Competing interests
The authors declare no competing interests.

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