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To cite this article: M Šijačic-Nikolic et al 2021 IOP Conf. Ser.: Earth Environ. Sci. 875 012002

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Conservation of forest genetic resources through the example of native *Quercus* species from the "Košutnjak" park forest in Serbia

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Abstract. Given the importance of urban park forests and protected natural areas in the urban environment, the implementation of the concept of genetic conservation is one of the main ways to save and improve this category of natural resources. Forests such as Košutnjak can be considered a refuge for flora and fauna and by establishing conservation programs for certain species, the entire ecosystem would be better protected. The purpose of this paper is to elaborate a systematic approach to in situ conservation of forest genetic resources in urban areas through the example of four native oak species: pedunculate oak, sessile oak, Hungarian oak and downy oak in the protected natural area "Košutnjak" in the capital of Serbia - Belgrade, to develop replicable conservation guidelines for urban park forests. The methodological framework for the development of guidelines for genetic conservation, presented in this paper, is a replicable model whose basic principles can be applied in other cases of urban park forests.

1. Introduction

Forest ecosystems are exposed to a continuous process of pollution and fragmentation, due to the implementation of other forms of land use [1]. Compared to other types of forest landscapes, urban park forests are exposed to a significantly higher level of anthropo-pressure, due to their specific location and environment [2]. The forest areas within the city have special value as refuge habitats of flora and fauna and areas of importance for the recovery of species and preservation of the natural integrity and optimal land use, as well as for improving the quality of the urban environment [3].

The *Quercus* species are one of the most important tree taxon across the northern hemisphere in terms of species diversity, ecological dominance, and economic value. Oaks are taxonomically perplexing because of shared interspecific morphological traits and intraspecific morphological variation, which are mainly attributed to hybridization [4]. Main threats to oak species, besides many insects and pathogens [5], are human pressure and the transformation of land into agricultural use [6], changes in rainfall distribution and incidence of stress-induced pathogens [7], relatively new declinedisease Acute Oak Decline Syndrom (AOD) affecting mostly Q. robur and Q. petraea [8], forest dynamic and the evolution of forestry practices (forsaking of coppicing, aging of the populations) and introduction of exotic genotypes through plantations [9].

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Forestry-2021	IOP Publishing
IOP Conf. Series: Earth and Environmental Science 875 (2021) 012002	doi:10.1088/1755-1315/875/1/012002

According to [10] Oak (*Quercus* spp.) is declining globally due to a variety of pathogens, pests, and climate change; and assessments of the impact of losing those species should include the impact on associated biodiversity and ecosystem functioning, and consider mitigation options. They assessed the potential ecological implications of a decline in *Quercus petraea/robur* within the UK and collated a database of 2300 species associated with *Q. petraea/robur*, and concluded that establishing alternative tree species would be one potential mitigating measure for lessening the impact of oak decline on associated biodiversity.

The goal of the review conducted by [11] was to compile a list of oak species of conservation concern, evaluate the available genetic data for these species, and highlight the gapst. Authors compiled a list of 124 Oaks of Concern based on the Red List of Oaks 2020 and the Conservation Gap Analysis for Native U.S. Oaks and their evaluations of each species. The results shown that 57% have been the subject of some genetic analysis, but for most threatened species (72%), the only genetic analysis was done as part of a phylogenetic study. Authors of this survey concluded that some Oaks of Concern have benefited from population genetic research, but most are lacking basic conservation-focused data, and the countries with the highest numbers of threatened oak species are China with 36, Mexico with 32, and the United States with 28.

According to [12] 45% of the *Quercus* species evaluated by IUCN are considered threatened (being affected by diseases and pests or habitat destruction) in the UK. Those authors noted that conservation of oaks is becoming increasingly important for many countries, but conventional seed bank strategies are not suitable for their long-term preservation because seeds are desiccation sensitive, while tissue culture is a useful tool for *Quercus* species conservation, supporting micropropagation and in vitro conservation. However, they concluded that cryopreservation is accepted as the most favourable and cost-effective option for long-term *ex situ* conservation of oaks and they reviewed the main cryobiotechnological options for the successful banking of species in the genus *Quercus*.

The conservation of genetic resources should be seen as efforts to preserve the specific genotypes or populations and the characteristic combination of genes in them [13, 14]. Given the importance of urban park forests and protected natural areas in the urban environment, as well as their vulnerability to current climate change, the implementation of the concept of genetic conservation is one of the main ways to save and improve this category of natural resources. Forest woody species in the urban environment, especially oaks as long-lived species with genotypes of imposing dimensions are one of the main categories for inclusion in conservation programs.

The purpose of this paper is to elaborate a systematic approach to *in situ* conservation of forest genetic resources in urban areas through the example of four native oak species: pedunculate oak (*Q. robur* L.), sessile oak (*Q. petraea* (Matt.) Liebl), Hungarian oak (*Q. frainetto* Ten.) and downy oak (*Q. pubescens* Willd.) ("rare-endangered" [15]) in the protected natural area "Košutnjak" in the capital of Serbia – Belgrade, to develop replicable conservation guidelines for urban park forests.

2. Methodology

2.1. An integrative methodological framework

Key steps for the development of a conservation program were recommended by Rotach [16], but given the specifics of urban park forests, a more detailed methodology has been developed that includes the following steps (figure 1):

- 1. Field survey and identification of woody plant species;
- 2. Field survey and identification of herbaceous plant species;
- 3. Assessment of the degree of endangerment of woody plant species;
- 4. Assessment of the degree of endangerment of herbaceous plant species;
- 5. An overview of rare, vulnerable, and endangered plant species, with an assessment of the degree of endangerment and defining priorities for conservation;
- 6. Survey of localities and individuals of the most endangered plant species;
- 7. Spatial definition and mapping of the most endangered plant species localities and genotypes;

- 8. Selection of mother trees within the group of the most endangered woody plant species;
- 9. Seed collection at the level of selected mother trees;
- 10. Estimation of mother trees variability applying morphological markers;
- 11. Estimation of mother trees variability applying molecular markers;
- 12. Establishment and maintaining of progeny tests in a nursery and/or at the field;
- 13. Estimation of the variability of different half-sib lines applying morphological markers;
- 14. Estimation of the variability of different half-sib lines applying molecular markers;
- 15. Defining *in situ* conservation measures for the most endangered trees;
- 16. Defining ex situ conservation measures for the most endangered trees;
- 17. Development of replicable conservation guidelines/program for managing urban park forest following the principles of conservation;
- 18. Establishing a monitoring system to follow the implementation of the guidelines, with special reference to the possibility of natural regeneration and *in situ* conservation;
- 19. Strengthening the capacities of managers for the implementation of the guidelines for genetic resources conservation and promotion of natural values of urban park forests.
- 20. Evaluation of the implementation of the guidelines after each programming period and according to the developed monitoring system.

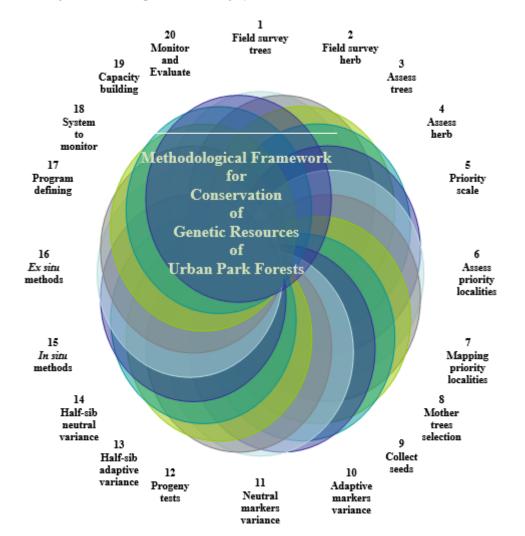


Figure 1. An integrative methodological framework for conservation of genetic resources in urban park forests.

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Such a comprehensive methodological framework has been applied, in order to be tested, in the pilot area of the Košutnjak park forest and protected natural asset in Belgrade, the capital of Serbia. The methodology has been tested on four native oak species: pedunculate oak (*Q. robur* L.), sessile oak (*Q. petraea* (Matt.) Liebl), Hungarian oak (*Q. frainetto* Ten.) and downy oak (*Q. pubescens* Willd.) ("rare-endangered" [15]).

2.2. Study area

Košutnjak park forest is located within the cultural and historical entity Topčider, on the territory of Belgrade's municipalities Čukarica and Rakovica (figure 2). It is located above the left bank of the Topčiderska River on a vast plateau, to which a lower slopes intersected by valleys are continuing. The slopes of the terrain are very steep and reach over 70%. The altitude of the area ranges from 75 masl to 216.9 masl [17].

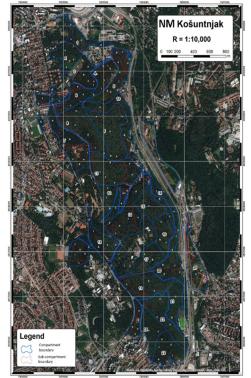


Figure 2. Map of the "Košutnjak" park forest.

"Košutnjak" is one of the two only remnants of the indigenous forest vegetation in the densely populated urban structure [18] and it is declared protected due to significant spatial functions and bioecological values of the complex under forest vegetation and to preserve the habitat of diverse fauna of mammals, birds, insects, reptiles and amphibians, as well as signs of geological discovery [17].

In "Košutnjak" forest ecosystems are predominantly broadleaf type, with a small proportion of the conifers. In this area, the presence of 521 plant species was recorded [2]. Given that 3730 species grow in Serbia, this number speaks of the high floristic diversity of "Košutnjak", which further supports the fact that it ranks third in the number of plant species in Belgrade when it comes to similar green complexes (after Avala and Kosmaj) according to the criteria of the diversity of vascular plants [19]. The largest part of Košutnjak forest (265 ha) is under the protection regime of the third (III) degree. The second-degree protection regime is present on the small part of "Košutnjak" – the area of the Natural reserve Forest of oak and hornbeam at Hajdučka fountain that has been protected since 1981 (3.5 ha) [18].

By studying the vegetation of "Košutnjak", six autochthonous oak species have been recorded so far: Turkey oak (*Q. cerris* L.), Oak of Virgil (*Q. virgliana* Ten.), downy oak (*Q. pubescens* Willd.), sessile oak (*Q. petraea* (Matt.) Liebl), pedunculate oak (*Q. robur* L.), Hungarian oak (*Q. frainetto* Ten.). Mesophilic hornbeam (*Carpinus betalus* L.), white lime (*Tilia tomentosa* Moench) and xerotherman manna ash (*Fraxinus ornus* L.), make the mesophilic, xeromesophilic, and xerothermic oak forests of today's Košutnjak.

3. Results and discussion

3.1. Previous application of the methodological framework

Fieldwork has been undertaken to fulfil the first 5 steps of the methodological approach. Inside the genus *Quercus*, especially subgenus *Quercus* (White oaks) is very difficult to determine different oak species with certainty due to extremely complex taxonomy, high species numbers, great morphological variability, and high rate of hybridization. The first step (1. *Field survey and identification of woody plant species*) included reconnaissance of the terrain and determination of oak species according to the selected morphological parameters (bark, buds, cupule and leaf) and ecological conditions in which the tree is located (figure 3).





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Figure 3. Representative oak genotypes recorded during fieldwork.

The second step (2. *Field survey and identification of herbaceous plant species*) included reconnaissance of the area and determination of the most important species of herbaceous plants. Within the third and fourth step (3. *Assessment of the degree of endangerment of woody plant species* and 4. *Assessment of the degree of endangerment of herbaceous plant species*), a preliminary assessment of the degree of endangerment of trees and herbaceous plants performed. Step five (5. *An overview of rare, vulnerable and endangered plant species, with an assessment of the degree of endangerment and defining priorities for conservation*) produced a very important output in the form of a review of all species of the importance for conservation.

In October 2019, a *Survey of localities and individuals of the most endangered tree species* (step 6) was conducted. In total, 330 trees were selected (80 pedunculate oaks; 81 sessile oaks; 81 Hungarian oaks, and 88 downy oaks) and every tree was geolocated using the UTM Geo Map application (Google

Play Apps) and labeled at breast height. Each selected tree was labeled with the letter: L – pedunculate oak; K – sessile oak; S – Hungarian oak; M – downy oak, and with an appropriate number (L01-L80; K01-K81; S01-S81; M01-M88) (figure 4) (step 7. *Spatial definition and mapping of the most endangered plant species localities and genotypes* and step 8. *Selection of mother trees within the group of the most endangered woody plant species*).



Figure 4. Selected individuals of pedunculated oak in the "Košutnjak" park forest as an example of the application of methodological steps 6, 7 and 8.

All four oak species are different in their bioecological characteristics, and it is difficult to point out areas in which they are equally distributed. Sites, where the number of trees is smaller, will not be singled out as separate conservation areas, but measures will be proposed at the level of individual genotypes or groups of trees. Seed collection at the level of selected oak mother trees was performed in autumn 2019 to establish generative progeny tests (step 9. *Seed collection at the level of selected mother trees*) (figure 5).



Figure 5. Appearance of downy oak fruits in different stages of formation.

After collection, seeds were separated from each tree to determine the weight of a thousand seeds. Step 10 (10. *Estimation of mother trees variability applying morphological markers*) has so far been

implemented for downy oak selected mother trees. The assessment included analysis of leaves, nuts, and cupulas morphometric traits (figure 6).

The leaves were collected from 41 selected trees and the analysis included 60 leaves per tree, the total number of measured samples is 2460. After collection, the leaves were herbarized and scanned. For the analysis of morphological traits, the methodology given by Franjić [20, 21] and adapted by [22] was used. The leaves were scanned by the *ImageJ* software.

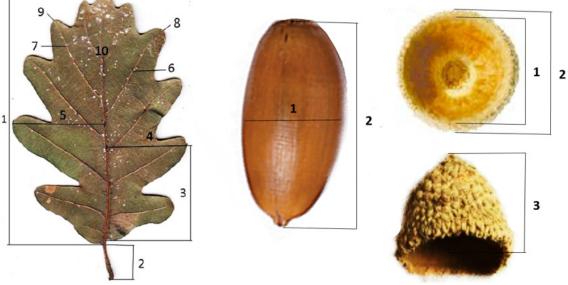


Figure 6. Morphological markers for downy oak leaves, nuts and cupulas. LEAF: 1 – lamina length (mm); 2 – petiole length (mm); 3 – lamina length (from leaf base to the widest part of leaf on the right side) (mm); 4 – lamina width from midrib to the widest part on the right side (mm); 5 - lamina width from midrib to the widest part on the left side (mm); 6 – number of veins on the right side; 7 – number of veins on the left side; 8 - number of lobes on the right side; 9 - number of lobes on the left side; 10 – the presence of leaf hairiness. NUT: 1 – nut width; 2 – nut length. CUPULA: 1 – cupula width inside; 2 – cupula width outside; 3 – cupula length.

To analyze the variability of morphological traits of downy oak nuts and cupulas, nuts from 20 trees (100 pieces per tree) with an abundant yield were separated. The obtained data were processed in the program *"STATGRAPHICS Centurion XVI*".Based on the obtained mean values, a cluster analysis was performed. Assessment of the variability of morphological markers of other oak species is planned for the next phases of the research.

Step 11 (11. *Estimation of mother trees variability applying molecular markers*) has been planned for all four oak species. This phase began with the collection of leaves from 80 genotypes per species. The material was prepared and stored and appropriate molecular markers were selected. The selection of nuclear microsatellites to be used to assess the molecular variability of four oak species present in Košutnjak was performed based on data from 327 scientific publications (for example, [23-27]). At the time of writing the paper, laboratory research is in progress in the Laboratory for Biotechnology of the Faculty of Forestry University of Belgrade: total genomic DNA isolation; spectrophotometric verification of DNA yield and purity in isolates; PCR amplification; electrophoresis; and determining the length of PCR amplification products. Bioinformatics processing of the obtained molecular data will be realized in the continuation of the research.

Steps 12 and 13 (12. *Establishment and maintaining of progeny test in a nursery and/or at the field* and 14. *Estimation of variability of different half-sib lines applying morphological markers*) has so far been implemented for downy oak selected mother trees. An assessment of the variability of the pubescent oak gene pool in the "Košutnjak" park forest was conducted at the level of 41 selected representatives, using morphological markers and progeny testing, established in a nursery of 20 half-

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sib lines, using morphological and physiological markers. High variability in the height and number of leaves was found in plants from different lines of half-sib progenies, which represents a significant contribution to knowledge on the variability of this species. Further research should continue at the level of different lines of half-sib progenies in the nursery and at the field [28].

3.2. Future application of the methodological framework

Further research includes continued analysis of molecular markers for selected mother trees as well as estimation of variability of different half-sib lines at the field and in the laboratory, applying morphological and molecular markers (14. *Estimation of variability of different half-sib lines applying molecular markers*). The establishment of the field trial is planned for the autumn of 2021, and in that way, a part of the gene pool will be conserved.

When all the analyzes have been performed and the results processed, steps 15 and 16 (*Defining* 15. *in situ and* 16. *ex situ conservation measures for the most endangered trees*) will be conducted. Preliminary definition of conservation units was performed based on field survey, but modification of the map based on variability analysis is expected (figure 7).

The final steps of the methodological approach will be to develop a replicable conservation guidelines/program for managing urban park forests based on the results from the "Košutnjak" park forest as a pilot area (step 17). The guidelines will contain a monitoring system model to follow the implementation of the guidelines, with special reference to the possibility of natural regeneration and *in situ* conservation (step 18).

After the completion of research and development of guidelines, strengthening of the capacities of managers for conservation and promotion of natural values of urban park forests will be conducted through trainings, workshops, and round tables (step 19).

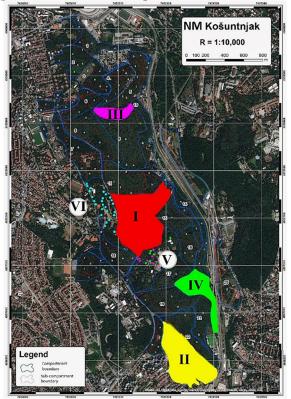


Figure 7. Preliminary conservation units for four oak species in the Košutnjak park forest.

Long-term evaluation of the implementation of the guidelines after each programming period and according to the developed monitoring system will be organized (step 20).

Conclusion

All mentioned above indicate the importance of conservation of all four species of native oaks in the "Košutnjak" park forest. Urban forests such as "Košutnjak" can be considered a refuge for flora and fauna and by establishing conservation programs for certain species, the entire ecosystem would be better protected.

The methodological framework for the development of guidelines for genetic conservation, which is presented in this paper through the example of the "Košutnjak" park forest, is a replicable model whose basic principles can be applied in other cases of urban park forests. This was the first time to propose such methodology for genetic conservation of "Košutnjak" park forest, and we presented one part of our comprehensive research. There is a need for future conservation activities in this area.

Certainly, this draft methodological framework cannot serve as a rule and be followed step by step without any changes. It primarily serves to indicate the complexity of a systematic approach to the development of conservation programs. The concept of activities requires modifications depending on the specific species or urban localities [13]. All conservation units, that will be defined in the final phase of the research, should be visibly marked, with an emphasis on easy-to-read and user-friendly information on the importance of biodiversity conservation and environmental protection.

Acknowledgments

This work was supported by the Secretariat for Environmental Protection of the City of Belgrade, project: *Identification and monitoring of the gene pool of rare, vulnerable and endangered plant species in NM "Kosutnjak Forest"* and Ministry of Education, Science and Technological Development of the Republic of Serbia according to the agreement number 451-03-9/2021-14/ 200169.

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