

Vieira Natividade Experimental Plots at Mata Nacional do Vimeiro, Portugal.

The Hybrids *Quercus x hispânica* 'Lucombeana' (*Quercus cerris x suber*)

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Abstract. In 1933, Joaquim Vieira Natividade established an experimental field with oaks and related genera at Mata Nacional do Vimeiro, Portugal. This experimental area includes the only Portuguese plots with the hybrid *Quercus x hispânica* hybrid 'Lucombeana' (*Quercus cerris x suber*) with seeds obtained from the Cambridge Botanic Gardens. In 1962-1965, their offspring were included in this area.

Those plots integrate an *ex situ* forest genetic resources, representing a unique population for understanding the structure, function and evolution of the genome (genomics) and its gene expression (transcriptomics). In all progenies, with approximately 60 years, it is evident the segregation for several phenotypic characteristics, specifically in the cork formation and in the leaf shape. Based on that, two projects were developed with this plant material. The first was focused on finding out the molecular basis responsible for the variation in cork and the other on association genetic studies.

The aim of this work is to allow access and sharing of knowledge about this genetic material in accordance with the established by FAO in the State of the World's Forest Genetic Resources report. Information about these hybrids is available at PANGAEA repository.

Key words: Forest genetic resources; genetic conservation *ex situ*; genetic conservation *in situ*; PANGAEA repository

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Parcelas experimentais de Vieira Natividade na Mata Nacional do Vimeiro, Portugal.

Os híbridos *Quercus x hispânica* 'Lucombeana' (*Quercus cerris x suber*)

Sumário. Em 1933, Joaquim Vieira Natividade estabeleceu um campo experimental com carvalhos e géneros afins na Mata Nacional do Vimeiro, Portugal. Esta área experimental inclui os únicos talhões com o híbrido *Quercus x hispânica* 'Lucombeana' (*Quercus cerris x suber*) obtidos com sementes provenientes dos Jardins Botânicos de Cambridge. Entre 1962-1965, as suas descendências foram incluídas nessa área.

Esses talhões constituem uma área de conservação genética *ex situ*, representando uma população única para estudos de investigação com vista ao conhecimento da estrutura, função e evolução do genoma (genómica) e a expressão dos genes (transcriptómica). Em todas as famílias aí representadas, com aproximadamente 60 anos, é evidente a segregação em diversas características fenotípicas, nomeadamente na formação da cortiça e na forma das folhas. Com base nisso, desenvolveram-se dois projetos utilizando esse material vegetal. O primeiro focou-se na análise das bases moleculares responsáveis pela variação da cortiça e o outro em estudos de associação genética.

O objetivo desta publicação é permitir o acesso e a partilha da existência deste material genético, de acordo com o estabelecido, pela FAO, no relatório sobre o estado dos Recursos Genéticos Florestais no Mundo. A identificação destes híbridos está acessível em repositório.

Palavras-chave: Recursos genéticos florestais; conservação genética *ex situ*; conservação genética *in situ*; repositório PANGAEA

Parcelles expérimentales de Vieira Natividade à Mata Nacional do Vimeiro, Portugal.

Les hybrides *Quercus x hispânica* 'Lucombeana' (*Quercus cerris x suber*)

Résumé. En 1933, Joaquim Vieira Natividade a établi un champ expérimental avec des chênes et des genres apparentés dans Mata Nacional do Vimeiro, au Portugal. Cette zone expérimentale comprend les seuls peuplements avec l'hybride *Quercus x hispânica* 'Lucombeana' (*Quercus cerris x suber*) obtenu à partir de graines du Cambridge Botanic Gardens. Entre 1962-1965, ses descendants ont été inclus dans ce domaine. Ces parcelles constituent une zone de conservation génétique *ex situ*, représentant une population unique pour comprendre la structure, la fonction et l'évolution du génome (génomique) et de ses expressions des gènes (transcriptomique). Dans toutes ces familles, âgées d'environ 60 ans, la ségrégation est évidente dans plusieurs caractéristiques phénotypiques, à savoir la formation du liège et la forme des feuilles. Sur cette base, deux projets ont été développés en utilisant ce matériel végétal. Le premier portait sur l'analyse des bases moléculaires responsables de la variation du liège et l'autre sur les études d'association génétique.

Le but de cette publication est de permettre l'accès et le partage de l'existence de ce matériel génétique, comme a été établi par la FAO dans son rapport sur l'état des ressources génétiques forestières dans le monde. L'identification de ces hybrides est accessible dans le référentiel.

Mots-clés: Ressources génétiques forestières; conservation génétique *ex situ*; conservation génétique *in situ*; dépôt PANGAEA

Introduction

The Mata Nacional do Vimeiro is located in the municipality of Alcobaça, Portugal (39°32'N; 8°58'W). This forest consists of four different areas: Mata da Roda, Mata da Ribeira, Mata do Canto and Mata do Gaio, all together occupying an area of 267 hectares. Prior to its integration into state property, it was known as Mata da Coutada or Gaio and belonged to the Alcobaça Monastery, at that time inhabited by the Cistercian monks (NATIVIDADE, 1929). In 1836, after extinction of the religious orders, it was incorporated into the General Administration of the Kingdom Woods and was subject to the forestry regime under decrees in 1901 and 1903 (ICNF, 2015). Currently, it is managed by the department Conservação da Natureza e das Florestas – Lisboa e Vale do Tejo (DCNF-LVT) of the Instituto da Conservação da Natureza e das Florestas (ICNF, I.P.).

According to the Regional Plan of Forest Management of the West (PROF-Oeste), this national forest has the following functions: wood production, environment protection and recreation. The main species are maritime pine (*Pinus pinaster* Aiton), cork oak (*Quercus suber* L.), Portuguese oak (*Quercus faginea*), chestnut (*Castanea* sp) and other hardwoods (Figure 1). It is a rugged relief forest with a small flatter area, the nursery, where currently is located an experimentation area established in the thirties of last century, the so called Vieira Natividade experimental plots (ICNF, 2015).

In 1930, Joaquim Vieira Natividade, agronomist and forester, was appointed as director of the Cork Oak Experimental Station which previously inaugurated in April 1924 by initiative of Mendes de Almeida from the Portuguese Forest Services. The main purpose was to investigate solutions for solving many practical problems that the cork oak culture raised. It was intended to: determine the causes of the decline in cork quality of the Portuguese Montados; to establish the scientific bases for regulating the most important cultural operations such as pruning, thinning, soil mobilization; to determine the technical conditions for the crop management to guaranty the best use for the cork oak lands (PEREDA, 2008). In fact, during his staying as director he elected the Mata Nacional do Vimeiro to carry out several field studies establishing experimental plots at the nursery area not only with the cork oak, but also with other species. These assays were established using different techniques: sowing, transplantation and vegetative propagation (cuttings, grafting, and layering). Ten years after the Cork Oak Experimental Station inauguration, NATIVIDADE (1941) presented the different domains that had been studied so far and the

results achieved, namely in the anatomy, histology, physiology, cytology and genetics of cork and reinforcing that the results allowed to know deeper the species and help guide cultural practices.

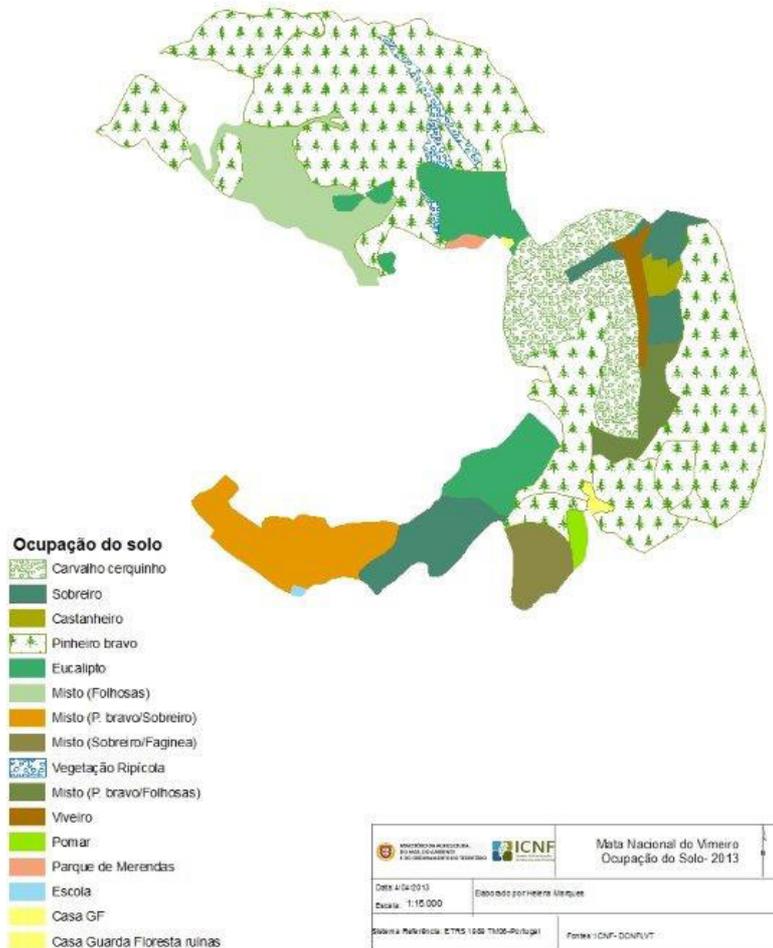


Figure 1 - Maps of species distribution over Mata Nacional do Vimeiro, Alcobaca, Portugal (2013) including the following species: Portuguese oak (Carvalho cerquinho, *Quercus faginea*), cork oak (Sobreiro), chestnut (Castanheiro), maritime pine (Pinheiro bravo), *eucalyptus* (Eucalipto), hardwoods (Folhosas) and riparian vegetation (vegetação ripícola) (Source: ICNF, 2015)

Natividade's interest in carrying out scientific research in the Mata Nacional do Vimeiro was already reflected in his final Forestry Engineer report (NATIVIDADE, 1929). He described this National Forest as having exceptional conditions for the study of hardwoods, namely the soil type. Later, in 1933, Branquinho de Oliveira, in a letter sent from Cambridge, also encourages Natividade to establish in Alcobaça an experimental field with oaks and related genera, referring to the importance of such field structure would have as a complement to the Cork Oak Experimental Station. He compared it to the existing structures of the internationally renowned Citrus Station in California from United States of America. He also emphasizes the importance that it would represent for the enhancement of the national heritage, as well as in a creation of a training school not only for those who wished to dedicate themselves to forestry life and scientific work but also for young forest engineers. While in Cambridge preparing his PhD, Branquinho de Oliveira also offers himself as an intermediary with the Cambridge Botanic Gardens to obtain seeds of interesting species to be installed in this possible experimental field (BRANQUINHO-OLIVEIRA, 1933). In fact, it must have been through his friend and colleague that Natividade obtained the *Quercus x hispanica* hybrid 'Lucombeana' (*Quercus cerris x suber*) seeds. He planted them in 1933 in Mata Nacional do Vimeiro nursery. This plant material is referenced in Natividade's technical-scientific works and of his collaborator Brito dos Santos. The cytological study carried out in some species and hybrids of the genus *Quercus* (NATIVIDADE, 1937), as well as the reference to the variability found in cortical cambium (NATIVIDADE, 1936) are two examples of studies done with this plant material. In fact, at that time Natividade was committed to carrying out cytological studies on several species of Mediterranean oaks (NATIVIDADE, undated). Being able to complete these studies using other oaks, such as hybrids obtained from crosses with oaks from different habitats seems to have motivated him to obtain the *Quercus cerris x suber* hybrid seed. Later, NATIVIDADE (1941) recognized the importance of those hybrids for genetic studies. Therefore, he outlined the cork oak genetic improvement plan considering three research lines related to hybridization.

BRITO-SANTOS (1944) in his study on bark segregation (bark and cork formation) and foliage (shape, stomatal density and pubescence) refers to these hybrids as being F3 offspring obtained from two oaks from the Cambridge Botanical Gardens, classified as typical representatives of the hybrid *Quercus cerris x suber*. The author concludes that there is no dominance in hereditary characters linked to the studied characteristics, cork formation and bark and foliage type, confirming what NATIVIDADE (1937) had already mentioned

regarding the foliage characters and showing a set of varied forms among the characteristics attributed to *Quercus suber* and *Q. cerris*. Later, in the 60s, half-sib progenies of these hybrids were sown in different places of the nursery. Nowadays, it can be observed the existence of trees with different degrees of cork formation and distribution along the trunk, showing the segregation of cork as a quantitative characteristic, that is, gene-regulated. The same segregation type was observed for the leaves.

The collection of segregating hybrids of *Q. x hispanica* trees constitutes an elite material for the development of several research and experimental studies. In fact, this hybrid *in vivo* collection is an example of a genetically unique population that should be included to a *ex situ* conservation program. The aim of this work is to allow access and sharing of knowledge about this unique genetic material. This objective is in accordance with FAO (2014) report which recommends that countries should improve the availability and accessibility of information systems (databases) about their forest genetic resources.

Experimental area at Mata Nacional do Vimeiro

Vieira Natividade experimental plots began to be established in the 1930s in a flat area of the Mata Nacional do Vimeiro nursery. They occupy a total area of 10.12 hectares and consist of seven different plots (Figure 2; Table 1). Cork oak is the main species in these plots but there are also other plots with chestnut and oak hybrids such as plot 5, and plots 6 and 21a, respectively.

Plot 18 was established in 1946 and is constituted of trees planted in lines. These cork oaks were transplanted from the Mata Nacional das Mestras, Caldas da Rainha, Portugal (39°28'N; 9°03'W) and until now, they are maintained without being stripped. Almost all the trees from plot 19 are ramets from a unique tree selected by Natividade in Mata Nacional das Mestras for its high cork quality. It was nicknamed as serralheira. The clone was obtained by bud-grafting and the ramets were planted in 1972. In plot 20, the cork oak coppice experiments were initially planted in 1951. Three years after plantation all plants were cut to promote the burst. The first evaluation of these experiments took place in 1962 (CORREIA, 1964). In plot 21, half of the cork oaks were obtained by vegetative propagation methods: cuttings, layering or mounding. In the other half, the seedlings were planted in a high forest production system using a spacing of 3 x 3 meters.



Figure 2 – Detail of Vieira da Natividade's experimental plots at Mata Nacional do Vimeiro, Alcobça, Portugal

Table 1 - Characterization of Vieira Natividade experimental plots

Plotnumber	Area (ha)	Species	Observations
5	1.94	<i>Castaneasativa</i> ; <i>C. crenata</i> ; <i>C. molissima</i>	Different chestnut varieties selected for fruit production (1980) and for resistant to ink disease (1950)
6	0.73	<i>Quercus x hispanica</i> Lucombeana (<i>Quercus cerris x suber</i>)	Hybrids obtained from seeds that came from the Cambridge Botanic Gardens (1933)
18	1.10	<i>Quercus suber</i> L.	Trees transplanted from Mata Nacional das Mestras (1946)
19	2.68	<i>Quercus suber</i> L.	Cork oak monoclonal bank propagated by grafting (1972)
20	1.17	<i>Quercus suber</i> L.	Cork oak in coppice system (1951)
21	3.00	<i>Quercus suber</i> L.	Cork oaks obtained from different propagation methods
21a	1.44	<i>Quercus x hispanica</i> Lucombeana (<i>Quercus cerris x suber</i>)	Half-sib progenies from the hybrids of the plot number 6 (1962-1965).

Hybrids *Quercus x hispanica* 'Lucombeana' (*Quercus cerris x suber*)

All the hybrids *Quercus x hispanica* 'Lucombeana' (*Quercus cerris x suber*) are installed in plots 6 and 21a at Vieira Natividade experimental field. In 1933, sixteen plants were established in plot 6, using seeds obtained from the Cambridge Botanic Gardens. At the moment, there are still thirteen hybrids surviving (Figure 3). Between 1961 and 1965, progenies of some of these hybrids (mother trees 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 14, 15 and 17) were installed in four different small sub-plots from plot 21a: 52, 53, 54 and 55 (Figure 4).

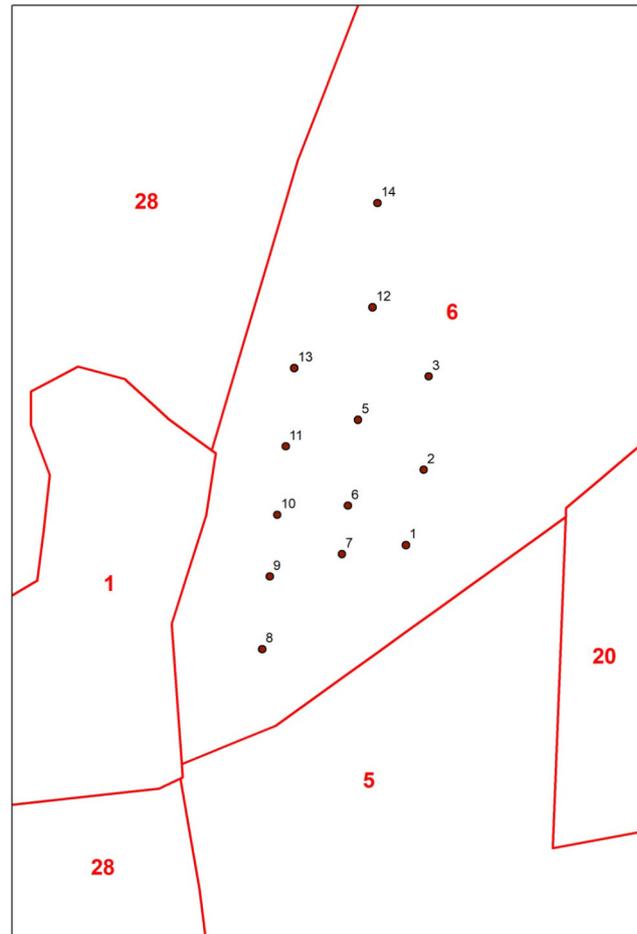


Figure 3 - Localization of the mother trees, the hybrids *Quercus x hispanica* 'Lucombeana' (*Quercus cerris x suber*) planted in plot 6



Figure 4 - Localization of the offspring of mother trees *Quercus x hispanica* 'Lucombeana' (*Quercuscerris x suber*) at sub-plots 52, 53, 54 and 55 (included in plot 21a).

During several years these mother trees were monitored for their phenological behavior such as flowering and fructification and some variability was observed. For example, every year, trees number 1, 2 and 8 produce seed. However, the feminine flower of the hybrid number 8 appears in the first year but its acorns only ripen in the second year, which is a typical behavior of *Quercus cerris* species.

In sub-plots 52-54, the plants were distributed in lines in a spacing of 2.5 x2.5 meters, according to their respective mother tree (Table 2). In sub-plot 55, the last one to be installed (1964 and 1965), the seeds were simply sown in a spacing 0.3 x 0.3 meters without identification of their parent origin. However, this sub-plot included progenies from mother trees 1, 2, 4, 7, 9, 11, 12, 14, 15 and 17 that were not represented in the sub-plots 52, 53 and 54 (Table 3).

Table 2 - Initial and actual number of hybrids per family in the sub-plots 52-54 of the plot 21a

Hybrid Mother	Sub-Plot	Total Number of Plants Initially Established	Total Number of Trees per Family (2019)
1	54	14	5
2	52, 53, 54	74	40
3	53	22	13
6	53	16	8
8	54	27	10
10	54	14	9

Table 3 - Total number of seeds per mother tree sown in 1964 and 1965, at the sub-plot 55 from plot 21a

Hybrid Mother	Total Number of Seeds Initially Sown
1	211
2	780
3	640
4	2
7	180
8	390
9	80
10	700
11	150
12	140
14	80
15	1
17	3

In 2019, all the surviving trees belonging to plots 6 and plot 21a, were identified and geo-referenced. For each of these hybrids, the geographical coordinates, total height and the diameter at breast height are deposited and available at PANGAEA Data Archiving & Publication (CARRASQUINHO *et al.*, 2020). From a total of 195 hybrids, 13 are the mother trees (plot 6) and 182 their offspring (plot 21a). Until now, the families of 85 half-sibs are identified (sub-plots 52-54). Considering to the hybrid's progenies, mother tree number 2 has the larger offspring, 40 trees, whereas hybrids 1 and 10 are less represented, 5 and 9 individuals, respectively. The individual family identification of the 96 half-sibs sown in sub-plot 55 is ongoing.

Research and experimental studies with the hybrids *Quercus x hispanica* 'Lucombeana' (*Quercus cerris x suber*)

In 2009, a National Portuguese initiative organized a Consortium to study cork oak Expressed Sequences Tags (ESTs). Within this Consortium, 12 projects were designed to obtain a deeper understanding of *Q. suber* functional genomics (PEREIRA-LEAL *et al.*, 2014). To take advantage of this hybrid population with different cork production levels, one of the Consortium projects, SOBREIRO/0017/2009 - Cork oak ESTs Consortium - Cork production, coordinated by INIAV was approved. This project was focused to find out the molecular basis responsible for the variation in the cork, using genomic (a set of genetic information) and transcriptomic (gene expression) approaches involving the use of high-throughput sequencing technology. At the transcriptome level, the first detailed comparison between cork producing and non-cork producing trees, revealed a significant number of genes differentially expressed and several mechanisms exclusively associated with cork production trees, which constitutes a major advance in our knowledge regarding the genetic regulation behind cork formation and production (MEIRELES *et al.*, 2018).

In 2015, another project was approved, PTDC/AGR-FOR/3356/2014 (Hybrid Oak Project) - Characterization of cork formation and reproductive biology in a cork oak hybrids population, coordinated by Instituto Superior de Agronomia, Lisbon University. The objective was the study of the parental genomes interaction in order to detect genes differentially expressed that can be responsible for some of the contrasting traits observed.

In summary and as it was initially proposed, it is now possible to access, share and use all these data for future studies. The sequence reads obtained

from transcriptomic studies were already deposited in the NCBI Sequence Read Archive (SRA) under the accession numbers ERX143070 and ERX143071, for the normalized libraries, and SRX2677031 and SRX2677030, for the non-normalized libraries. These results will greatly enhance the knowledge and understanding of cork formation and production processes. Information about the living hybrids, such as the location (geographic coordinates), female parent identification, sowing year and biometric characterization (total height and breast height diameter) are also available at PANGAEA repository (CARRASQUINHO *et al.*, 2020).

Acknowledgments

The authors gratefully acknowledge to Direcção-Geral do Património Cultural/Mosteiro de Alcobaça for providing access to Casa Museu Vieira Natividade documental archive, i.e., the archive belonging to Joaquim Vieira Natividade. The authors also thank to Associação de Produtores Florestais dos Concelhos de Alcobaça e Nazaré (APFCAN) for providing trees geographic coordinates and the Figures 2-4.

This study was financed by Fundação para a Ciência e a Tecnologia (PT) (SOBREIRO/0017/2009 - Cork oak ESTs Consortium and PTDC/AGR-FOR/3356/2014 - Hybrid Oak).

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