PhD THESIS

Characterization of genetic variability of chestnut in Romania

(SUMMARY OF PhD THESIS)

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INTRODUCTION

Woody species in the national forestry fund are found in different forest formations, forming stands of a varied structural and functional complexity.

Although the structure of the national forestry fund is carried out by a relatively small number of species or groups of species, having as reference elements the area they cover and the volume of standing trees, there are a number of species considered at various or disseminated category that have ecological and economic importance.

The chestnut (*Castanea sativa* Mill.), although it is found insular in the national forestry fund in some representative locations, has a particular importance for the forestry sector, so, the culture of this species can be extended both in the forestry fund and beyond it. Also of particular interest is the agro-forestry systems in which chestnut crop may be of particular interest, given the climate changes of the past decade, at regional, continental and planetary level, aspect that facilitates the culture of this species promotion on considerable areas.

This species is important both for its valuable wood, resistant to various harmful agents and for its fruit with high nutritional value, represented by chestnuts. Cultural and economic value of stands depends on forest site potential, their composition and not at least, by the manner of administration and management.

Promoting in the future the chestnut species in the stands in which it vegetates now, is an objective for the local forestry administration, in order to conserve the flora and fauna biodiversity and, respectively, for optimal exploitation of forest resources. Although, at the present, at national level, there is a certain interest in chestnut culture, in both forestry and in some research and development horticultural stations, studies on the genetic structure and diversity of chestnut populations, as well as its distribution and its origin, using modern investigative methods, are relatively modest.

Considering the possibilities for research and analysis of various genetic markers related populations of trees and shrubs using molecular markers, it is now possible to conduct a comprehensive study on the populations of chestnut in Romania, taking into account the achievements, similar in this area, at European level. Also, of particular importance, is the research of pollen (which is based on the study of fossil pollen preserved in peat bogs, related to different species) that have been done in our country and at European level, in recent decades.

PhD thesis structure

The PhD thesis contains a number of 135 pages, 47 figures, 34 tables and 29 photos being structured in two main parts, namely Literature review and Personal contribution.

Part I Literature review consists of 4 chapters:

Chapter 1 General considerations concerning the chestnut (*Castanea sativa* Mill.) refers to the presentation, taxonomy and ecology of the genus *Castanea*, as well as the forestry of chestnut.

Chapter 2 Methods and techniques for assessing genetic diversity using markers include generalities about using markers to assess genetic diversity, types of genetic markers and techniques used to highlight molecular markers.
Chapter 3 Forest genetic resources at national level presents general aspects of the gene pool, genetic resources conservation in situ and ex situ and forest genetic resources established under in situ and ex situ conservation cores, included in the national catalog and regions of origin of chestnut species.

Chapter 4 Studies and research concerning the chestnut species refers to the palinological studies, modern researches and those concerning chestnut culture, made at European level and in Romania.

Part II Personal contributions include 6 chapters:

Chapter 5 Research purposes and objectives

Research purposes
The purpose of the research theme is the study of genetic variability of chestnut (Castanea sativa Mill.) populations in Romania, in order to implement a strategy for conservation and sustainable management of forest genetic resources.

Research objectives
- identification and sampling of chestnut populations in Romania;
- identification and sampling of chestnut populations in Belasitsa Massif, Bulgaria;
- presentation of current areal of the species;
- description of spatial structure of chestnut stands studied;
- highlighting existing haplotypes chloroplast genome (ADNcp) by PCR-RFLP technique, in the chestnut populations studied;
- evaluation of genetic diversity of intra- and inter-population inside chestnut populations studied;
- evaluation of inter-population and intra-population genetic diversity at the nuclear genome level of Castanea sativa Mill. populations in Romania, using SSR markers;
- identifying populations with high diversity indices (ex. allelic richness, rare alleles);
- identifying genetic barriers within populations of chestnut in Romania;
- delimitation of areas of origin based on population genetic structure;
- following the completion of the all the studies concerning the current distribution of populations, spatial structure of forest stands, genetic structure and genetic diversity of the population, it is necessary to formulate recommendations on sustainable management and the dynamic conservation of the gene pool of chestnut population from Romania;
- delimitation of areas of origin on the basis of the population genetic structure will help to avoid "genetic pollution" into the national gene pool of chestnut;
- there are necessary certain recommendations concerning expanding into crop of the species, both in the forestry fund and also in the agroforestry systems or in other kind land.

Chapter 6 Environmental peculiarities in which the case study was conducted includes location of case study and vegetation conditions related to the chestnut stand studied.

The chestnut stands from the national forestry fund that is the subject of the case study, where from samples were collected (sprouts with buds), are found within the area of Gurahonţ Forest District - Arad County Forest Administration, Dobreşti Forest District - Bihor County Forest Administration, Tismana Forest District and Runcu Forest District - Gorj County Forest Administration, Baia de Aramă Forest District and
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Tarnița Forest District – Mehedinți County Forest Administration, Baia Sprie Forest District, Tăuții Măgherăuș Forest District and Firiza Forest District - Maramureș County Forest Administration, Codrii Cămării R.A. Forest District - Dobrești City Hall. For achieving the established objectives it was studied a natural population of chestnut from the Belasitsa area - Southern Bulgaria.

Chapter 7 Material și method

Biological samples were collected from 18 populations of chestnut from Romania and one natural chestnut population from Belasitsa. The sampling was done outside the vegetation season and consisted of 30-40 cm length sprouts with buds, that were collected from the same specimen, aged over 20 years. The vegetal material was analyzed genetically by means of PCR-RFLP method using universal primers for the amplification of the chloroplast genome: DT, FV and K1K2, developed by DEMESURE (1995) and DUMOLIN-LAPEGUE (1997); and the nSSR method using specific primers locus nuclear: CsCAT1; CsCAT2; CsCAT6; CsCAT16; CsCAT3; CsCAT14; EMCs38, developed by BUCK (2003); DANIELA Marinoni (2003). The extraction of total DNA, the testing of DNA quality and also the amplification and analysis of some regions of the chloroplastic DNA with the method of separation of fragments through electrophoresis (PCR-RFLP) were conducted in the laboratory of forestry genetics from the National Institute for Research and Development in Forestry Marin Drăcea - Department Simeria. The amplification of polymorphic DNA regions and analysis of PCR products by the method nSSR were conducted in the laboratory of Instituti di Biologia Agroambientale e Forestale, Consiglio Nazionale delle Ricerche, Italy under the coordination of Mrs. dr. Claudia Mattioni and the results were subsequently transmitted for statistical analyzes and interpretations.

Chapter 8 Results and discussions

8.1. Distribution and spatial structure of stands studied

At the present, in our country, chestnut it is distributed mainly in the northwest, the Baia Mare area (Baia Sprie, Firiza, Tăuții Măgherăuș Forest Districts), the Western area Gurahonț and Codrii Cămării – Dobrești (Gurahonț, Dobrești and Codrii Cămării Forest Districts) and in the Southern Subcarpathians area respectively in Baia de Arama - Tismana plateu and Călimănești in Vâlcea County (Baia de Aramă, Tarnița, Tismana, Runcu, Călimănești Forest Districts).

Fig. 1 The spatial distribution of chestnut populations studied in Romania
The spatial structure of chestnut stands (populations) from which the vegetal material was collected in order to carry out genetic analysis, using molecular markers, was made using PROARB 2.1 program. *Castanea sativa* Mill. species is represented in the profiles obtained with yellow and the tree from which the sprouts were collected was striped. For the genetic analyzes based on molecular markers were collected biological samples from the tree no. 7.

**8.2. Assessment of the genetic diversity with PCR-RFLP method**

After analyzing and processing the samples, a series of results were obtained regarding the identification of polymorphisms and haplotypes by PCR-RFLP technique. Determination of chestnut haplotype was achieved by measuring the polymorphic bands and taking into account the definition of the haplotype indicated by SILVIA FINESCHI (2000). Also, the numbering of the bands was carried out according to the method used by Silvia FINESCHI (2000).

**DT primer**

After separation of the fragments generated by cutting with the restriction enzyme Taq I, resulted 5 bands of different sizes. The polymorphism was detected in the bands 2 and 3, in each of the bands being detected two distinct fragments.

**FV primer**

The region of chloroplastic DNA amplified using the primer FV was cut through digestion, using the restriction enzyme *Hinf I*, resulting fragments 8 and 9, which are visible in the bands formed on the polyacrylamide gel. The polymorphism in the case of fragments from region FV was identified in bands 1, 2, 4, 5 and 8.

**K₁K₂ primer**

The last region analyzed by PCR-RFLP method relates to the flanked by the K₁K₂ primer, which by cutting, using a restriction enzyme Taq I, generated in the electrophoresis gel, 8 and 9 distinct fragments. Also, in the case K₁K₂ primer are clearly distinguished the two haplotypes, the polymorphism being evident. Even if the number of bands generated by cutting with restriction enzyme is the same, the lack of some bands or conversely, the existence of the others, makes it possible to clearly distinguish between the haplotypes.

**Chloroplast genetic diversity of chestnut populations in Romania and Bulgaria**

Analyzing the map of fig. 3 it is noted also that the spatial distribution of chestnut populations studied, it is highlighted the presence of two distinct haplotypes. For all populations from Romania it is established the existence of a single haplotype - haplotype I, and in Belasitsa Mountains, from the south of Bulgaria, it is highlighted the presence of two haplotypes, one identical to that of Romania - haplotype I and the other different from the first, noted haplotype B.
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For achieving a comprehensive analysis, from the point of view of the genetic diversity of chestnut populations using ADNcp, there were used the results of research conducted and published by SILVIA FINESCHI (2000). Thus, there were analyzed and interpreted unitary the results obtained, being produced in this respect a map of the spatial distribution of haplotypes associated to the chestnut populations studied in Europe.

Fig. 3 The spatial distribution of haplotypes associated to the chestnut populations studied in Romania and Bulgaria

It is found that the haplotype associated to the populations of chestnut in Romania and one of the two identified in Bulgaria (haplotype I) is found in the north-west Turkey, southern and central Italy, southern France and most part of the Iberian Peninsula. The haplotype identified in most populations in Belasitsa Massif, and different from that from Romania (haplotype B), is also found in other areas of Europe, respectively southern and western Turkey, Central Italy and the Iberian Peninsula.

8.3. Assessment of the genetic diversity with nSSR method

All the 7 SSR locus analyzed were polymorphic, being detected in total 102 allelic varieties. The number of alleles per locus identified varies from alleles 5 (locus CsCAT14) at 26 alleles (CsCAT3 locus). CsCAT1 locus indicates the least degree of heterozygosity observed and expected (H₀, Hₑ) and locus CsCAT3 the highest degree of heterozygosity observed and expected (H₀, Hₑ).

The inbreeding coefficient (Fᵢₛ) is positive and significant (p <0.05) only locus CsCAT2 same observations were noted by ILARIA LUSINI (2014). The inbreeding coefficient values (Fᵢₛ) were positive for most populations, but not significant (p> 0.05), with the largest positive values in Tarnita (Fᵢₛ = 0.12) and Gurahonț-5 (Fᵢₛ = 0.11).

Allelic richness (R) represents a very important diversity index, it can indicate priority populations for conservation, measuring the relative abundance observed allelic variants in populations with equal number of individuals. Maximum values of allelic richness index were identified in populations from Codrii Cămării-2 (R=6.5), Dobrești-1 (R=6.27) and Gurahonț-5 (R=6.26), while the lowest values were observed in populations from Tarnita (R=3.77) and Baia de Aramă (R=3.83).
The average value of genetic differentiation coefficient ($F_{ST}$) between the population of chestnut 16 is moderate respectively 12.45% (P=0.001), which may be because of the existence of barriers of gene flow. The high degree of genetic differentiation was observed between populations Baia Sprie and Tarnița ($F_{ST}$=0.199), Baia Sprie and Runcu ($F_{ST}$=0.198), Baia Sprie and Tismana-1 ($F_{ST}$=0.198). The lowest degree of differentiation was evidenced between populations Baia Sprie and Firiza ($F_{ST}$=0.018).

The STRUCTURE analysis, described by EVANNO (2005) highlighted the most likely two major genetic groups, according to the maximum values for $\Delta K$=2. The degree of difference between the two genetic groups revealed high values of the differentiation coefficient $G'_{st}$ (Hed) = 0.641 (P=0.002).

Hierarchical structure in populations of chestnut was confirmed by analysis of the dendrograms obtained with NJ method and UPGMA algorithm.
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BARRIER program has detected the existence of a discontinuity gene flow between the two genetic clusters identified with Bayesian analysis. The barrier identified in assessing genetic discontinuity between chestnut populations analyzed, divides them into two distinct groups. How the method at spatially level assesses to discontinuities based on genetic differences, can say that northern populations studied have evolved separately from those studied in western, southwestern and southeastern Romania.

Priority populations for conservation have been established by parameter allelic richness values - Rs for each genetic cluster. For the cluster in western Romania, the highest value regarding allelic richness is found in populations of the Codrii Cămării-2 (Rs=6.50) and it is considered as a priority for conservation. Referring to the cluster in the southern part of the country, the population of the Călimăneşti present priority for conservation because allelic richness has the highest value (Rs=4.99). According to size of the red points there were materialized geographical areas which are a priority for conservation, respectively, the higher the red point is, the higher the priority for conservation is.

In parallel with this case study, it was conducted a genetic analysis for populations from 73 geographic areas, situated in ten countries from Europe and Asia, at Instituto di Biologia Agroambientale e Forestale. From Romania there were selected three populations from different areas respectively, one population from Dobreşti area, one from Baia Sprie area and one from Tarniţa area. The results obtained are found in an article that was submitted for review for publication in the Journal Tree Genetics and Genomes.
Chapter 9 Conclusions and recommendations

General conclusions

It is noted that, by using primers DT, FV and K₁K₂, following the interpretation of the results, it was highlighted a single haplotype I for all chestnut populations analyzed from Romania.

It is also found that by using primers DT, FV and K₁K₂ in analyzing samples of chestnut from Belasitsa Massif located in Southern Bulgaria, it was highlighted the presence of two haplotypes, one denoted haplotype I, that is identical to that from Romania and respectively haplotype B, different from the first.

Considering this case study and the researches conducted by Fineschi et al. it is concluded that the haplotype associated to the populations of chestnut in Romania and one of the two identified in Bulgaria is the best represented by identifying 79% of the samples analyzed, being encountered in the north-west Turkey, southern and central Italy, southern France, and most of the Iberian Peninsula.

It is also observed that the other haplotype, identified in Belasitsa Mountains from the south of Bulgaria, it also exists in the southern and western Turkey, Central Italy and the Iberian Peninsula.

For a suggestive representation of haplotypes of Castanea sativa Mill. identified at European level, was used an application related to geographic information systems, particularly important aspect in context of the unitary analysis for the studied chestnut populations.

From the spatial distribution analysis of the 11 haplotypes identified at European level by the method RFLP, it can be concluded that the populations of chestnut in Romania could have originated in northwestern Turkey and/or Italy and, not at least, could be considered a possible glacial refuge.

For the first time in Romania it was assessed allelic richness and degree of heterozygosity of chestnut populations using 7 genetic markers, the type of repetitive sequences, nuclear microsatellites (nSSRs).

The highest number of allelic variants were identified in population Dobreşti-1 with 49 allelic variants and Codrii Cămării-2 with 48 allelic variants.

The lowest number of allelic variants were detected in the population from Baia de Arama and Tarnița with 28 allelic variants.

From the analysis of the population structure made with two clusters (K = 2) it is found that the populations of chestnut in Romania are grouped into two genetically distinct groups, most likely, due to the different postglacial origin and/or anthropogenic influences (colonization by man with material genetic from populations that come from two different glacial refugia).

Based on analysis of the genetic structure of the populations made with three clusters (K = 3) it was found the existence of three groups genetically distinct, two groups (in the Carpathian area) are more closely related genetically, and the third group (extra-Carpathian area) is isolated, being detected the existence of a genetic barrier with the other two genetic groups.

The carrying out of the applications relating to the studied structure stands in the horizontal, vertical and 3D plane, highlights synthetically structural particularities of the stands in which the chestnut vegetates and from which the vegetable material was collected.

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Recommendations

According to the obtained results, it is recommended the delimitation of three regions of origin for basic material to be made up into forest reproductive material of chestnut:

- the first region of origin to be composed of the following extra-Carpathian populations: Baia de Aramă, Tarnița, Tismana Runcu and Călimănești;
- the second region of origin to be composed of the following populations: Gurahonț, Dobrești and Codrii Cămării;
- the third region of origin include the following populations: Tăuții Măgherăuş Firiza and Baia Sprie.

It is recommended the creation of new forest genetic resources representative for each genetic group detected:

- for the extra-Carpathian is proposed the population of Călimănești because it is located at south-eastern limit of the area of distribution in Romania;
- the inclusion in the National Catalogue of Forest Genetic Resources a chestnut population in northern areal distribution respectively population from Baia Sprie or Firiza that have a distinct and homogeneous gene pool;
- the inclusion of chestnut populations from Codrii Cămării and Gurahonț in the National Catalogue of Forest Genetic Resources, the population from Dobrești is already included.

Conservation of representative chestnut populations in each area studied, taking into account the value of the parameter genetic allelic richness. It is worth mentioning that the genetic center ("hot spot") of chestnut in Romania is composed from the populations from Codrii Cămării, Dobrești and Gurahonț where it was identified maximum values of allellic richness parameter.

One important challenge in the molecular genetics, but also forestry domain, would be the identification and genetic fingerprinting of resistant forms of chestnut pathogens, respectively *Cryphonectria parasitica* and *Phytophthora sp.*, in order to extend their crop.

Establishing effective and efficient criteria for the selection and promotion of chestnut genotypes in stands (crops) of national forestry fund and in a crops from the agro-forestry systems.

Promoting the future of chestnut stands by natural regeneration from seed under massive in situ thus preserving local provenance.

Realizing of genetic analysis for age classes in chestn populations, obtained artificially or by natural regeneration (seed or sprouts and/or suckers) in the studied areas for establishing provenance and conservation priorities.

Considering the results it is recommended to carry out complex research that can elucidate origin of chestnut populations studied.

Expanding genetic analyzes in crops of chestnut in other geographical areas in our country where it vegetates and which were not analyzed in this study.

Extending the application of treatments in disease prevention for chestnut in all populations affected by them (microinjections with phosphite acid against *Phytophthora* species and using strains hipovirulente of *Cryphonectria parasitica* in chestnut bark cancer treatment).

The ecological reconstruction of stands affected by biotic factors, respectively *Cryphonectria parasitica* and *Phytophthora sp.*, using local biological material.
(seedlings and/or chestnuts obtained from stands located in said areas or neighboring areas).

Realizing of spatial databases at national level with stands seed sources and respectively, seed reserves of appropriate the chestnut, following mappings carried out rigorously, to consider the phenotypic characteristics of populations (stands) of chestnut and results of genetic analyzes made in these.

Chapter 10 Innovative contributions of the thesis

In this thesis is approached the first genetic analysis using molecular markers of populations of chestnut which vegetates in the forestry fund. As a result, there were used two consecrated methods PCR-RFLP and nSSR to investigate the genetic structure and diversity of populations of chestnut in Romania.

Through PCR-RFLP method it was identified for the first time the haplotype I related to chestnut species for the populations from Romania, that is usually encountered in most of the populations of Europe.

As a result, the genetic analysis using nSSR technique and interpretation of the results with specialized programs were obtained for the first two distinct clusters corresponding chestnut populations from Romania of which one is divided into two sub-clusters relatively different from each other.

Establishing for the first time of a possible barrier of gene flow between chestnut populations studied from Romania and its materialization using geographic information system map.

Also, according to the genetic parameters obtained from the interpretation of the results there were identified and established the priority chestnut populations for conservation.

Were analyzed integrated the palinological studies achieved so far in our country with the results of genetic analyzes performed in order to establish the possible hypotheses of the post-glacial evolution of the populations of chestnut from Romania.

Usage of geomatic applications for spatial positioning of the populations studied and reporting the results in a georeferenced system at national and European level.

Presentation and description of the chestnut areal in Romania, as well as, making a synthesis of the studies and existing researches at European and national level regarding this species.

Description of populations and stands of chestnut in Romania and Belasitsa Massif-Bulgaria from which it was collected biological material for genetic analysis with molecular markers.

Conclusions to be drawn on the structure, diversity of populations and possible post-glacial evolution of chestnut from Romania.

Elaboration of recommendations on conservation of chestnut populations from Romania, their inclusion in the National Catalogue of Forest Genetic Resources, and the possibilities of extending and promotion in crop, both in the national forestry fund as well beyond.
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