

Recent developments in the selection and breeding programme on *Juglans* spp. in Italy

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ABSTRACT

Introduction

Only 20% of Italy's hardwood timber requirements are met from domestic markets.

Noble hardwoods are very important for the Italian furniture industry, being one of the most advanced for design and technology in Europe. Approximately 150,000 ha have been planted with noble hardwoods since 1985 with 70% of these represented by wild cherry, walnuts and oaks. In the last ten years, the estimated turnover for seed supply alone exceeded 50 million €.

Two main ways are therefore possible to supply wood for the Italian industry: importing wood, mainly from France and Balkan countries (3.5 millions of cubic meters are imported yearly) and developing programmes to improve their stock in forests.

In the framework of several research programmes, CRA-ISSEL and CNR-IBAF assessed the genetic variation of walnuts (practically unknown earlier) and established a network to preserve, select and improve basic genetic resources suitable for the above mentioned aims.

Activities and results of improvement strategies for walnut (*Juglans regia* L.) are presented..

Studies for Walnut (*Juglans regia* L.) seed orchards models

Walnut in Italy has been the focus of a substantial research effort since 1985 concentrating on breeding and improvement of the species. The priority has been initially preserving what remains of the *in situ* genetic resource after intensive felling. No natural populations of the species are present in any part of Western Europe.

Variations was assessed initially throughout Europe by neutral (Isozymes, RAPDs and SSRs) and adaptive markers, as phenology and frost resistance/avoidance.



Concerning *Juglans regia*, phenotypes suitable for timber production have needed to be developed, such as straight stem, branch architecture, dominance and resistance/tolerance to pests and diseases. Methods were based essentially on a multi-trait Selection Index to start recurrent selection by progenies.

The present step is concluding evaluation of selected materials and the possibility to transform field test into a seedling seed orchards is being explored.

Phenotypically superior trees were grafted and stored in clone archives. Therefore progeny a tests were planted in the most suitable environments in order to evaluate their *genotype x environment* interaction. A multi-trait *Selection Index* method for progeny and phenotype evaluation was found to be very effective.

Studies were then undertaken to assess the genetic variation related to even more strict selection criteria. As for many forest tree species the individual genetic component was found to be higher than at the inter-population level. Within the different models of orchards tested genetically, the progressive reduction of progenies did not significantly modify the levels of variation compared to the whole field test used and the reference Italian populations. Homozygosity levels are in general higher in walnut than in other species. Wright's F values decrease across different selection intensities when selection is carried out by progenies, and increase when individual selection is carried out.

Homozygosity was lower within the selected progenies models than in the others. In this study, four selected progenies showed greater allelic richness. A hypothetical seed orchard made with these progenies at the same time could provide material with good performance and supply variability similar to larger populations such as the total plantation or the reference pseudo-natural system.

Phenotypic, Biochemical and Molecular Markers for study a interspecific a new hybrid walnut population in northern Italy

Whilst the above researches were carried out a friendly competition with French Colleagues was devoted to look possible parental trees to produce hybrids *J. nigra x regia*. After several years a promising mixed population was discovered in northern Italy and is being studied and reproduced vegetatively to establish a specific nursery system in order to produce hybrid seedlings.



In the framework of the genetic resources valorisation, this study aims to select, characterise and to test for reproduction interspecific hybrids (*J.nigra x regia*) of walnut to be used in reforestation programmes and for genetic improvement. Those hybrids are known as *Juglans* × *intermedia* Carr.; they are also of horticultural value being an important rootstock.

Recently, detection of specific DNA sequence differences (cpDNA for the maternal lineage and ITS regions for the fact of hybridisation) have been applied for distinguishing species and hybrid trees. Studies of walnut isozymes, DNA by RAPD and ISSR markers are well-known. Since nuclear SSRs (microsatellites) are codominant DNA markers, they are highly suitable for use in population genetic studies, genome mapping, tree breeding, DNA genotyping/fingerprinting of important clones, and as a consequence of the usual heterologous amplification possibility within a genus, they are also a practical tool for the detection of hybridisation and parentage analysis.

Our work has been developed to compare different types of nuclear DNA markers – RAPD, ISSR and SSR - with respect to their effectiveness in distinguishing the parental species and hybrid progeny within the population.

Plant material

The sampled plants are originated from a small population growing within a private park near the County of Vicenza, Venice region, Italy. A total of 138 individuals include 69 samples initially classified (by phenotype characteristics) as *J. nigra* (N3 - N5, N17, N18, N21 - N23; NC4, NC6 - NC11, NC13, NC15, NC16, NC19, NC21 - NC24, VR1 – VR46), 48 samples identified as *J. regia* (R6 – R16; B2 – B20; V1 – V17; plus 1 tree of unknown origin), and 21 putative interspecific hybrid plants (H1, H2, H19, H20; IMP1, IMP3 – IMP7; IMP9 – IMP15; IMP17 – IMP20).

Methods

Genomic DNA was extracted by the method of Doyle and Doyle (1987) properly modified for *Juglans*. Amplification reactions both for RAPD and ISSR markers were performed in a GeneAmp 9700 PCR system following the appropriate procedures. The amplified fragments were separated by electrophoresis on agarose gel, stained with ethidium



bromide and visualized by under UV lamp. SSR markers were analysed by Biosystem Genetic Analyser 3100. Primers set up for *Juglans nigra* L., were provided by Keat Weste, Purdue University (UK).

Cytological analysis Developing male flowers from the selected trees were collected in a stage putatively young enough for the cytological examination. Developing pollen mother cells were microscopically checked for meiotic chromosome pairing after traditional acetic-carmine staining (by Agnes Major, Univ. Budapest).



Data analysis

For ISSR and RAPD data (dominant markers), the band presence/absence binary matrices were compiled. The comparisons were performed on the basis of SM (simple match) and Jaccard's similarity indices. For SSRs, the genotypes of the individuals were determined and their relationships were analysed first calculating the individual genotypic distances according to the squared size difference between two alleles (Slatkin, 1995), secondly computing the individual pairwise squared multilocus genotypic differences (Smouse and Peakall, 1999). Genetic diversity (R_{ST} and Φ_{PT}) was measured (Slatkin, 1995; Smouse and Peakall, 1999) and AMOVA analysis was carried out. The statistical analysis of the data was performed by GenAEx V5.1 (Peakall and Smouse, 2003) and NTSYSpc2.1 (Rohlf, 2001) softwares.

Results and Conclusion

Both dominant (188 RAPD, 162 ISSR bands) and codominant markers (113 SSR alleles) reliably discriminated the individuals according to their origin. Mantel's tests proved that the multivariate approaches by these three molecular marker types described the same genetic structure of the two *Juglans* species and their interspecific hybrid progeny. SSR markers resulted the more appropriate for the analysis of hybrid plants. During genotyping the 138 investigated trees we found that one individual (N21), originally classified by RAPD and ISSR markers as an exemplar of *J. nigra*, showed the presence of three alleles at 6 microsatellite loci. These genotypes indicated the **triploid hybrid** nature of this individual showing 2/3 *J. nigra* alleles and 1/3 *J. regia*. The triploid hybrid nature of N21 was confirmed by the cytological analysis.

The highly variable microsatellite loci will be ideal for DNA fingerprinting, pedigree analysis and parentage testing for hybridogenic populations. The comparison between the multilocus genotype of the putative progenies and the *J. nigra* plants allowed detecting, among the others, a putative mother trees giving rise to the major part of hybrids. Paternity analysis is still in progress. In this framework two grafted copies of the whole basic population were established.

The future activities will be devoted to establish a network of progeny field tests which will be mainly used to study paternity and to select new hybrid sibs.



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