Characterization of walnut genotypes for production of hybrids: a case study in Italy

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Juglans regia L.
(Common walnut, persian walnut, English walnut)

Juglans nigra L.
(Black walnut)

1- Michigan; 2- Ohio; 3- Indiana; 4- Illinois; 5- Missouri; 6- Kentucky; 7- Tennessee; 8- West Virginia; 9- Virginia; 10- North Carolina; 11- South Carolina; 12- Georgia; 13- Alabama; 14- Kansas; 15- Iowa 16- Minnesota; 17- Wisconsin

[Map showing distribution and origin of walnut species]
After 1492 animals and plants were exported from America to Europe and vice versa.

Beginning in the 17th century, J. nigra was imported from United States to Europe for ornamental purposes.

Thus common and black walnut were in touch and, in particular conditions, they produced interspecific hybrids.
Know how

In Villa Mezzalira, a population including *J. nigra* and *J. regia* plants and some individual, naturally generated, growing in the undergrowth faster than others.

Hypothesis

The presence in the park of natural hybrids and therefore hybridogenic parents was supposed.

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They show vigor, faster growth than *J. regia* (especially in low and medium fertility soil), apical dominance, thin branches, late bearing, spring frost tolerance (Fady B. et al. 2003).

Flooding and drought tolerance in comparison with *J. regia*. (Mapelli S. et al. 1997)

Tolerant for bacteriose, anthracnose and bark cancer (?) (Anselmi N. Ri.Selv.Italia Sottoprogetto 1.1).

Wood production in relatively short time. (Prog. UE “W-Brains” FAIR III)

Interspecific hybrid *J. x intermedia* is the walnut prototype for quality wood production (Garavel L. 1972)

**Controlled crosses are difficult**
To obtain hybrids breeders should establish orchard with selected parent trees

Phenological observation (timing of pollen shed overlapping stigma receptivity)

Problems

- Adult trees in natural condition are often too high for phenological observations
- Long time (some year) to observe the overlap of flowering on grafted plants
- Long time to select hybridogenic mother and father trees
- The selection of hybrids is time consuming

Identification of interspecific Hybrids (NG23 X RA)

Morphological markers:

- Shape of fruits and leaves
- Position of the buds at the base of the collar
- Presence or absence of bracts at the base of the bud. (Jay-Allemand C. et al. 1990)

1 – opposite buds
2 – sub-opposite buds
3 – alternate buds
4 – bract at the base of the bud

J. nigra
J. regia
J. x intermedia
Objectives

Selection of hybrid plants obtained from free pollination in natural condition

Identification of the mother hybridogenic trees

Identification of the putative father(s)

 Establishment of seed orchard using selected plants for hybrid production

Two steps:

• 1) To acquire knowledge about the population

• 2) Research and selection of mother(s) trees

Father(s) identification
**Materials**
(CLASSIFIED ON THE BASE OF MORPHOLOGICAL CHARACTERS)

In the nursery “Peri” (Montecchio Precalcino) plants from seeds collected in the park 2 years before

- 46 *J. nigra* (VR);
- 17 putative *J. X intermedia* (IMP)

Total:
138 sampled plants

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**Molecular Markers**

Random Amplified Polymorphic DNA (RAPD): PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence

Inter Simple Sequence Repeat (ISSR): Casual amplification of genomic DNA regions, located among two repeated sequences oriented in opposite position, by single primers containing a short and a long microsatellite arbitrary sequence (anchor)

Microsatellites (SSR): Amplification of a genomic DNA fragment, containing microsatellite repeated sequences, by known primers complementary at flanking sequences of the target region.

The number of SSRs is highly variable among individuals
Selection and amplification of 10 primer SSR* 
113 amplified alleles in 138 samples

Sequence analysis of 10 microsatellite loci
Comparison with the consensus sequence— J. nigra available in the public database NCBI (BLASTn)

*SSR primers set up for J. nigra by K. Weste, Purdue University, CA, USA

Principal Coordinate Analysis based on Simple Matching (SM) similarity coefficient

188 RAPD bands
162 ISSR bands
113 SSR bands
Identification of hybrids (2n e 3N) (Locus SSR WGA 202)

Hybrids

J. NIGRA

N21

J. REGIA

N20

H1

H2

H19

Number of somatic chromosomes: 2n = 48 = 3x
Genomic composition: NNR

Maternity analysis (for exclusion)

<table>
<thead>
<tr>
<th>Maternità Putative</th>
<th>WGA321</th>
<th>WGA331</th>
<th>WGA49</th>
<th>WGA69</th>
<th>WGA9</th>
<th>WGA202</th>
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</thead>
<tbody>
<tr>
<td>N3     J. nigra</td>
<td>186</td>
<td>186</td>
<td>250</td>
<td>262</td>
<td>230</td>
<td>205</td>
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<tr>
<td>N4     J. nigra</td>
<td>186</td>
<td>186</td>
<td>250</td>
<td>262</td>
<td>230</td>
<td>205</td>
</tr>
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<td>N5     J. nigra</td>
<td>186</td>
<td>186</td>
<td>250</td>
<td>262</td>
<td>230</td>
<td>205</td>
</tr>
<tr>
<td>N17    J. nigra</td>
<td>188</td>
<td>186</td>
<td>250</td>
<td>262</td>
<td>230</td>
<td>205</td>
</tr>
<tr>
<td>N18    J. nigra</td>
<td>188</td>
<td>186</td>
<td>250</td>
<td>262</td>
<td>230</td>
<td>205</td>
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<tr>
<td>N22    J. nigra</td>
<td>188</td>
<td>186</td>
<td>250</td>
<td>262</td>
<td>230</td>
<td>205</td>
</tr>
<tr>
<td>N23    J. nigra</td>
<td>188</td>
<td>186</td>
<td>250</td>
<td>262</td>
<td>230</td>
<td>205</td>
</tr>
<tr>
<td>N21    Triploid</td>
<td>188</td>
<td>186</td>
<td>181</td>
<td>250</td>
<td>244</td>
<td>258</td>
</tr>
<tr>
<td>H1     Diploid</td>
<td>186</td>
<td>181</td>
<td>250</td>
<td>265</td>
<td>247</td>
<td>243</td>
</tr>
<tr>
<td>H2     Diploid</td>
<td>188</td>
<td>181</td>
<td>250</td>
<td>265</td>
<td>247</td>
<td>243</td>
</tr>
<tr>
<td>H19    Diploid</td>
<td>186</td>
<td>181</td>
<td>250</td>
<td>265</td>
<td>247</td>
<td>243</td>
</tr>
<tr>
<td>IMP3   Diploid</td>
<td>186</td>
<td>181</td>
<td>250</td>
<td>265</td>
<td>247</td>
<td>243</td>
</tr>
<tr>
<td>IMP4   Diploid</td>
<td>186</td>
<td>181</td>
<td>250</td>
<td>265</td>
<td>247</td>
<td>243</td>
</tr>
<tr>
<td>IMP9   Diploid</td>
<td>186</td>
<td>181</td>
<td>250</td>
<td>265</td>
<td>247</td>
<td>243</td>
</tr>
<tr>
<td>IMP18  Diploid</td>
<td>186</td>
<td>181</td>
<td>250</td>
<td>265</td>
<td>247</td>
<td>243</td>
</tr>
<tr>
<td>H20    J. regia</td>
<td>191</td>
<td>191</td>
<td>263</td>
<td>273</td>
<td>247</td>
<td>243</td>
</tr>
</tbody>
</table>

Common

Madri Putative

Ibridi

J. nigra

J. regia

IMP3

IMP4

IMP18

Common

Madri Putative

Ibridi

J. nigra

J. regia

IMP3

IMP4

IMP18

Common

IMP3

IMP4

IMP18
J. nigra N17 is really a hybridogenic plant?
In the garden is it the only mother?
The triploid is sterile?
Who is the candidate father? Is it only one or more?
…and if they are more, the reproductive success is the same?

Collection and plantation of the progenies;
Propagation of the hybridogenic mother trees

Propagation by graft (calls stimulated using hot tubes)
Fingerprinting of 8 half-sib families with 10 SSR loci

Half-sib families:
- Progeny N3: 118
- Progeny N4: 26
- Progeny N17: 88
- Progeny N18: 24
- Progeny N21: 17
- Progeny N22: 15
- Progeny N23: 59
- Progeny N24: 114

Total: 461

Assignment of the 461 genotypes to the defined 4 groups: J. nigra, J. nigra_NC, J. regia and hybrid.

Principal Coordinate analysis based on SM coefficient obtained with 119 SSRs

Assignement of the 461 genotypes to the defined 4 groups: J. nigra, J. nigra_NC, J. regia and hybrid.

Test di assegnazione

<table>
<thead>
<tr>
<th>Genetic Group</th>
<th>Number</th>
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<tbody>
<tr>
<td>J. nigra</td>
<td>328</td>
</tr>
<tr>
<td>J. nigra_NC</td>
<td>15</td>
</tr>
<tr>
<td>J. regia</td>
<td>49</td>
</tr>
<tr>
<td>Hybrids</td>
<td>205 + 3</td>
</tr>
</tbody>
</table>

N21-14
Locus WGA 276

N21-15
Locus WGA 321

Population Assignment

J. nigra 328
J. nigra_NC 15
Hybrids 205 + (3)
J. regia 49

Principal Coordinate analysis based on SM coefficient obtained with 119 SSRs

138 plants +461 progenies = 600 samples

Freiburg 6-9 September 2008
Maternal analysis of 8 half-sib families

Exclusion method + Most likely method

<table>
<thead>
<tr>
<th>Half-sib family</th>
<th>Putative progeny</th>
<th>Mother checking</th>
<th>J. nigra</th>
<th>hybrid</th>
<th>% hybrid</th>
</tr>
</thead>
<tbody>
<tr>
<td>N3</td>
<td>118</td>
<td>41</td>
<td>41</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N4</td>
<td>26</td>
<td>29</td>
<td>29</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N17</td>
<td>88</td>
<td>97</td>
<td>29</td>
<td>68</td>
<td>70</td>
</tr>
<tr>
<td>N18</td>
<td>24</td>
<td>15</td>
<td>15</td>
<td>0</td>
<td>0</td>
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<tr>
<td>N21</td>
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<td>18</td>
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<td>15</td>
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<tr>
<td>N22</td>
<td>15</td>
<td>73</td>
<td>73</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N23</td>
<td>59</td>
<td>74</td>
<td>57</td>
<td>17</td>
<td>22,9</td>
</tr>
<tr>
<td>N24</td>
<td>114</td>
<td>114</td>
<td>14</td>
<td>100</td>
<td>87,7</td>
</tr>
</tbody>
</table>

Paternal analysis  Most likely method (ML)

Exclusion method based on the probabilities of Mendelian segregation

CERVUS ver. 2.0

A

Only a putative father cannot be excluded

External pollination could happen

Assignation of the paternity is possible

B

Several putative fathers cannot be excluded

The paternity should be fractioned (external pollination included)

Paternity assignment is possible (probability)

C

All putative fathers should be excluded

We should enlarge the sampling area
Paternal analysis in the families

Reproductive success
Pollination for single mother
Summary of the obtained result (2 years)

Identification of 3 mother *J. nigra* plants producing interspecific hybrid *Juglans x intermedia* and their rate of generation:N23 (22.9 %), N17 (70 %)*, N24 (87.7 %)*

Identification of *J. regia* fathers with different reproductive success: V15, B6, B7, B13, B17

Early characterization of hybrid genotypes

Identification of a fertile triploid hybrid (N21) giving rise at diploid hybrids (83.3%), *J. nigra* individuals, and genotypes showing chromosomal errors.

Conclusion

Molecular markers, in particular SSRs, are a powerful and fast tool to approach practical research aspect, in this case early classification of germplasm.

They can be successfully utilised for:

• Forest nurseries for woody plants
• Seed orchards with characterised plants
• Comparative clonal tests and hybrids (*i.e. to test the reduction of genetic variability in collection and seed orchards.....*)

Functional genomics ..........
Thanks for your kind attention!

Hoping in a future and fruitful cooperation with you.

Freiburg
6-9 September 2008
J. regia is more susceptible than J. nigra to abiotic factors (flooding, drought) and biotic stresses, including anthracnose and bacteriosis.

Apical dominance; Flooding "resistance"; Growth faster than J. regia;

Quality Wood
Quality fruits

"Le noyer" Monographie 1999, E. German, J.P. Prunet, A. Garcin Eds.

J. nigra plantation 31 ys
(Eastern US Hardwoods, Douglass Jacobs)

Quality Wood Freiburg 6-9 September 2008

N21-14 locus WGA 276

N21-14 locus WGA 1

N21-15 locus WGA 321

N21-15 locus WGA 321