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# Retrospective identification of hybridogenic walnut plants by SSR fingerprinting and parentage analysis

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**Abstract** *Juglans* × *intermedia* (*Juglans nigra* × *Juglans regia*) is considered the prototype walnut for quality wood production in Europe. Hybridization between the parental species is rare under natural conditions and difficult using controlled pollination because of phenological and genetic incompatibilities. The identification of hybridogenic parents is the first step toward obtaining hybrid progeny. We report the application of microsatellite markers for DNA fingerprinting and parentage analysis of half-sib families collected in a natural mixed population for which no phenological and morphological data were available. Ten nuclear, neutral, simple sequence repeat markers were used to analyse 600 samples. The high levels of polymorphism detected positively influenced the exclusion and identity probabilities. The assignment analysis revealed the presence of 198 diploid *J.* × *intermedia* hybrids among the seedling progeny. Maternity checks were performed on all individuals and few errors of sampling were found. Four distinct hybridogenic mother trees were identified, each showing different reproductive success rates. The 198 diploid hybrids belonged to four open-pollinated families based on an analysis of paternity using a likelihood approach. Differential male reproductive success was observed among pollen donors within the research site. Forty-nine of the 198 diploid hybrids detected in four progenies were sired by only three *J. regia* genotypes. Backward selection might be used to establish new seed orchards for inter-specific $F_1$ hybrid production using genotypes with demonstrated compatibility.

**Keywords** *Juglans nigra* · *Juglans regia* · Hybrids · Microsatellites · Fingerprinting · Parentage analysis

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## Introduction

The importance of intra- and inter-specific hybridization for the genetic improvement of forest trees has been evident for at least 50 years (Zobel and Talbert 2003). Nevertheless, tree improvement often

has been narrowly focused on selection and breeding within a single native species. As suggested by Schreiner (1960), inter-specific hybridization also provides the maximum genetic diversity needed for greatest genetic improvement. Sometimes inter-specific hybrids may be difficult to obtain, however, even with the use of controlled pollination. This is the case for hybridization between *Juglans nigra* L. and *Juglans regia* L. that produces *Juglans* × *intermedia* Carr. Compared to the parental species, most *J.* × *intermedia* hybrids show increased vegetative vigor, distinct disease resistance, high wood quality, and greater winter-hardiness than *Juglans regia* (Fady et al. 2003). Consequently there is great demand for *J.* × *intermedia* for forestry, especially in Northern Europe.

Generally, the female parent of *J.* × *intermedia* is *J. nigra* and the male is *J. regia*. The difficulty obtaining hybrids of the two species could be the result of an incompatibility in flowering phenology or some mechanism(s) of genetic incompatibility (Sartorius 1990) such as inadequate chromosome pairing in megaspore and microspore mother cells (McKay and McKay 1941), failure of fertilization (pre-zygotic factors) or embryo abortion (post-zygotic factors). In addition to synchrony of flowering, hybridization rate may be affected by air temperature, which influences pollen germination and penetration through the stigma and the style to the *J. nigra* ovary. Luza et al. (1987) found clear differences in temperature optima for pollen germination and tube grow in *J. nigra* and *J. regia*.

The identification and selection of "hybridogenic" parents is the first step toward obtaining hybrid progeny. Although some trees are hybridogenic under natural conditions, it has been difficult to produce hybrids using controlled crosses (McKay 1965; Scheeder 1990). Breeders have encountered difficulties obtaining sufficient common walnut pollen at the time *J. nigra* pistillate flowers are receptive. No suitable and simple method for pollen storage and viability testing is available for *Juglans* (Luza and Polito 1985, 1988a, b). In addition, pistillate flower abscission (PFA), caused by excessive pollen load, has been reported in Persian (McGranahan et al. 1994) and black walnut (Beineke and Masters 1977). PFA may decrease the final nut set.

Seed orchards for hybrid production generally deploy one plus tree as a female parent and several plus trees as males to ensure enough pollen pressure. The oldest and best known European *J.* × *intermedia* (NG23 × RA) was obtained in France by the open-pollination of the female *J. nigra* NG23 with four *J. regia* plus trees RA984, RA996, RA331, RA295 as male parents (Becquey 1990). However, selection by phenological observations and clonal (graft) propagation of hybridogenic parent trees required more than 10 years (Jay-Allemand et al. 1990). Recent increases in industrial demand for wood have led to expanded planting areas and the establishment of new seed orchards for production of *J.* × *intermedia* trees. Application of the short-term marker-assisted breeding tactic developed by Grattapaglia et al. (2004) in *Eucalyptus* spp. could reduce the time needed to identify hybridogenic parents and to produce new hybrid genotypes in walnut. Grattapaglia et al. (2004) carried out SSRs-based paternity tests of superior hybrid progeny (*E. grandis* × *E. urophylla*) in forest stands established with open-pollinated seeds from an orchard. Parents displaying the highest reproductive success rates were identified, and a retrospective selection of parents was carried out.

Over the last 6 years in the framework of the national Project RI.SEL.ITALIA, the CNR. Institute of Agro-environmental and Forest Biology (Porano) has been intensively evaluating walnut germplasm in Italy. As a result of these efforts, a promising mixed population, including *J. nigra, J. regia* and some *J.* × *intermedia* hybrids, was discovered in Northern Italy and analyzed by SSR markers (Pollegioni et al. 2009). In this study, we report the detailed analysis of half-sib families collected in this population with the specific objectives of (1) detecting the presence of *J.* × *intermedia* in these progenies, (2) identifying *J. nigra* mother trees that spontaneously crossed with *J. regia* (hybridogenic mothers), and (3) verifying the differential reproductive success (DRS) of *J. regia* male parents (hybridogenic fathers) for production of hybrid offspring genotypes. By using backward selection after parentage analysis we hope to establish new seed orchards for hybrid production quickly using genotypes with demonstrated compatibility.

## Materials and methods

### Plant material

We examined a mixed population, including *J. nigra, J. regia* and some *J.* × *intermedia* plants growing in the Veneto region, Villa Mezzalira Park, Bressanvido (Northern Italy 45°39′0″N, 11°38′0″E). A total of 139 plants were sampled and previously genotyped using SSR markers (Pollegioni et al. 2009). DNA fingerprinting analysis classified 49 *J. regia* individuals, 82 *J. nigra* genotypes, 7 diploid *J.* × *intermedia* plants and one triploid hybrid showing two parts *J. nigra* genome and one part *J. regia*. Forty-nine *J. regia*, 8 *J. nigra*, 3 diploid hybrids, and a triploid (N21) are adult trees growing in the Park (Table 1; Fig. 3); 15 *J. nigra* adults plants (*J. nigra* NC) were located outside the park; 59 *J. nigra* and 4 diploid hybrids were 6-year old plants grown at the Veneto regional nursery (Montecchio Precalcino, Vicenza) from seeds collected in the Park. Parentage analysis of the trees in the Park indicated that *J. nigra* N17 was the hybridogenic mother plant of the seven diploid hybrids (Table 1) (Pollegioni et al. 2009).

In October 2005, seeds were collected from seven adult *J. nigra* trees and the triploid hybrid plant in Villa Mezzalira Park. The *J. nigra* N5 tree died and no offspring genotypes were available. The seeds were planted in a field atthe CRA Institute for Silviculture (Arezzo), and eight open-pollinated progenies (461 seedlings) were obtained: 41 seedlings from plant N3; 29 from N24; 88 from N17; 24 from N18; 76 from N22; 71 from N23; 114 from N24, and 18 from the triploid N21 (Table 3). In July 2006, mature leaves were sampled from each plant, immediately frozen in liquid nitrogen, and then held at −80°C until this analysis. In total 600 walnut samples were obtained.

### DNA extraction

Genomic DNA was extracted from all 600 samples including 461 progeny and 139 plants (already genotyped) for analysis. Samples were prepared for DNA isolation by grinding 100 mg of leaf tissue in a 2-ml microcentrifuge tube containing a 5 mm steel bead. The leaf tissue was homogenized in a Mixer Mill 300 (QIAGEN) cooled with liquid nitrogen. Genomic DNA was extracted and purified using the DNeasy96 Plant Kit (QIAGEN) according to the manufacturer's instructions (http://www.qiagen.com), and stored at −20°C. DNA quantity was assessed by comparing all samples against six standardized solutions of $\lambda$ phage DNA (15, 31, 63, 125, 250, 500 ng/μl; Life Technologies), in a 1% agarose gel stained with ethidium bromide and visualized with UV light. The DNA in the samples was brought to a working concentration of 5 ng/μl.

### Microsatellite analysis

Ten microsatellite loci (WGA1, WGA4, WGA9, WGA69, WGA89, WGA118, WGA202, WGA276, WGA321, WGA331) already sequenced and used for the preliminary genetic characterization of *J. nigra*, *J. regia* and *J.* × *intermedia* (Pollegioni et al. 2009) were amplified in all samples. The reproducibility of

**Table 1** Characteristics of 139 plants sampled in Villa Mezzalira Park, Bressanvido (Northern Italy 45°39′ 0″N, 11°38′0″E) previously genotyped using SSR markers (Pollegioni et al. 2009)

| Species | Group | Adult Trees (N) | Genotype label | 6 year old Trees[b] (N) | Genotype label | Total |
|---|---|---|---|---|---|---|
| *J. nigra* L. | *J. nigra* N | 8 | N3, N4, N5, N17, N18, N22, N23, N24 | 59 | N25-N83 | 67 |
| | *J. nigra* NC[a] | 15 | NC1-NC15 | – | – | 15 |
| *J. regia* L. | *J. regia* | 49 | R6-R16, A.E., B2-B20, V1-V17 | – | – | 49 |
| *J.* × *intermedia* Carr. | Diploid hybrid | 3 | H1, H2, H19 | 4 | IMP3, IMP4, IMP9, IMP18 | 7 |
| | Triploid hybrid | 1 | N21 | – | – | 1 |
| Total | | 76 | | 63 | | 139 |

[a] Fifteen black walnut adults plants located outside the park were labeled *J. nigra*-NC

[b] 6-year old plants growing at the Veneto regional nursery (Montecchio Precalcino, Vicenza), Italy, from seeds collected inside the park

the SSR markers was verified by comparison with samples already genotyped. Polymerase chain reaction (PCR) was performed using 20 ng of DNA template, 10 mM Tris–HCl (pH = 8.0), 50 mM KCl, 1.5 mM $MgCl_2$ reaction buffer, 200 µM dNTP (each), 0.2 µM primer (both), 0.008 µg BSA and 0.4 U of Taq polymerase (Roche Applied Science). Total reaction volume was 20 µl. Reactions were performed in a GENEAmp 9700 Thermocycler according to the following procedure: an initial denaturation at 94°C for 5 min, followed by 35 cycles of 45 s at 94°C, 45 s at the optimum annealing temperature for each couple of primes, and 1 min at 72°C; then a final extension step at 72°C for 7 min. A 5 µl of the amplified fragment was checked by electrophoresis in 1.8% agarose in 0.5× TBE buffer, and stained with ethidium bromide. To determine the exact size of the amplified microsatellite fragments, samples were diluted up to 1:10 in water and 1 µl of the diluted PCR product was mixed with 0.3 µl of a 500 bp internal-lane size standard (Gene Scan$^{TM}$ −500 ROX, Applied Biosystem) and 9.7 µl of pure deionized-formamide, denatured in a thermocycler at 95°C for 5 min, and immediately chilled on ice. PCR amplification fragments were resolved by capillary electrophoresis with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystem) (Table 2).

The amplified SSR fragment data were collected using Gene Scan Analysis version 3.7 Software and genotype profiles were assigned with the Genotyper version 3.7 NT Software (Applied Biosystem). In this study the typical co-dominant expression of SSR markers was complicated by the triploid state of some samples. Therefore, alleles were scored and recorded in a presence/absence matrix (0/1).

## Data analysis

On the basis of this binary code the simple match's similarity coefficient (SM—Sokal and Sneath 1963) was calculated between all pairwise combinations of individuals in order to evaluate the genetic relationships between genotypes. We used Principal Coordinate Analysis (PcoordA) based on the SM matrix to display the relative genetic distances among the 600 genotypes in a bi-dimensional plot (Rohlf 2001, NTSYSpc version 2.1 software package). After removing three triploid samples from the matrix raw data, diploid hybrid identification was definitively performed by assigning the offspring genotypes to one of the four classes (*J. nigra* N, *J. nigra* NC, *J. regia* and *J.* × *intermedia*). Two assignment tests were conducted using GENECLASS 2 software (http://montpellier.inra.fr/CBGA/softwares/): the Paetkau et al. (1995) frequency method, and Rannala and Mountain's (1997) partial Bayesian method. Both approaches removed the individual being assigned (leave one out procedure), computed the allelic frequencies in all candidate populations (assuming the HWE), calculated the likelihoods of the individual's multilocus genotypes occurring in each population (independence of loci), and assigned the individual to the population with the highest likelihood. Missing alleles were assigned anarbitrary nonzero frequency (0.01). The Rannala and Mountain (1997) method is comparable with the frequency method, but uses a Bayesian approach to estimate the allele frequencies of the population. In this study we applied these two tests incorporating, in addition, the exclusion-simulation approach of Cournet et al. (1999) to obtain a confidence level for each individual assignment (*P*-value = 0.01). The statistical threshold was calculated simulating 1,000 genotypes by the novel Monte Carlo resampling method (Paetkau et al. 2004).

Standard genetic diversity parameters, number of alleles per locus ($N_a$), effective number of alleles ($N_e$), observed ($H_o$) and expected ($H_E$) heterozygosity were calculated at each locus and over all loci for all 139 adult trees and the 459 offsprings assigned to the four classes. Departures from Hardy–Weinberg expectations at each locus were tested by a Chi-square test (Hedrick 2000). In studies requiring individual identification (DNA fingerprinting), the power of SSR markers to identify individual should be quantified. The unbiased probability of identity ($PI_{unb}$), the probability that two unrelated trees drawn at random from a population would have identical genotypes at multiple loci, was computed according to Paetkau et al. (1998). In highly sub-structured populations and especially in populations containing many large families, the theoretical equation of $PI_{unb}$ could underestimate the true probability of finding identical genotypes. Therefore, we also calculated the probability that two randomly selected full-sibs would exhibit identical genotypes ($PI_{sib}$) following the formula by Evett and Weir (1998). All calculations were performed using GenAlEx version 6 software (Peakall and Smouse 2005).

**Table 2** List of ten nuclear microsatellites previously sequenced and used for the preliminary genetic characterization of *J. nigra*, *J. regia* and *J.* × *intermedia* (Pollegioni et al. 2009). For each SSR the sequence, the repeat length, the annealing temperature and the Genebank accession number are shown

| Locus SSR | Primer sequence (5′ → 3′) | Repeat motif | Ta[a] (°C) | GeneBank accession number |
|---|---|---|---|---|
| WGA1 | F: ATTGGAAGGGAAGGGAAATG | $(GA)_5GCA(GA)_3GCA(GA)_3$ | 53 | AY465952 |
| | R: CGCGCACATACGTAAATCAC | | | |
| WGA4 | F: TGTTGCATTGACCCACTTGT | $(GT)_5(GA)_{15},(GA)_{11}$ | 53 | AY465953 |
| | R: TAAGCCAACATGGTATGCCA | | | |
| WGA9 | F: CATCAAAGCAAGCAATGGG | $(GA)_{16}$ | 55 | AY465954 |
| | R: CCATTGGTCTGTGATTGGG | | | |
| WGA69 | F: TTAGATTGCAAACCCACCCG | $(GA)_4ATATAA(GA)_{16}$ | 58 | AY333953 |
| | R: AGATGCACAGACCAACCCTC | | | |
| WGA89 | F: ACCCATCTTTCACGTGTGTG | $(GT)_{13}(GA)_{21}$ | 55 | AY352440 |
| | R: TGCCTAATTAGCAATTTCCA | | | |
| WGA118 | F: TGTGCTCTGATCTGCCTCC | $(GA)_{18}(GT)_{11}$ | 55 | AY479958 |
| | R: GGGTGGGTGAAAAGTAGCAA | | | |
| WGA202 | F: CCCATCTACCGTTGCACTTT | $(GA)_{20}$ | 58 | AY479959 |
| | R: GCTGGTGGTTCTATCATGGG | | | |
| WGA276 | F: CTCACTTTCTCGGCTCTTCC | $(GA)_{14}$ | 63 | AY479961 |
| | R: GGTCTTATGTGGGCAGTCGT | | | |
| WGA321 | F: TCCAATCGAAACTCCAAAGG | $(GA)_{14}$ | 53 | AY479962 |
| | R: TGTCCAAAGACGATGATGGA | | | |
| WGA331 | F: TCCCCCTGAAATCTTCTCCT | $(GA)_{13}$ | 53 | AY479963 |
| | R: CGGTGGTGTAAGGCAAATG | | | |

[a] The annealing temperature

## Assignment of parentage

Maternity of the *J. nigra* trees in eight families was first checked by the simple exclusion method, based on the Mendelian rules of inheritance. Paternity analysis for each diploid hybrid offspring (the assignment of a *J. regia* father to previously determined *J. nigra* mother—*J.* × *intermedia* offspring pair) was carried out using CERVUS version 2.0 (Marshall et al. 1998), a software based on the maximum-likelihood method (Meagher 1986). The haplotype of the putative male parent of each offspring was inferred by subtracting the female contribution from its multilocus genotype. The male haplotype was then compared with all possible haplotypes corresponding to the 49 *J. regia* adult trees in the Park. The maternal data were included to estimate selfing rate. Paternity was assigned to the male with the highest log-likelihood ratio (LOD score) among non-excluded males. The likelihood ratio represents how much more likely it is that the alleged father, rather than an arbitrary male, passed his genes to the offspring (Aitkin 1995). If two or more males were equally likely, paternity was deemed unassigned. Marshall et al. (1998) defined a Δ- statistic to assess the statistical confidence in the paternities that were assigned; where Δ value corresponded to the difference in LOD scores between the most-likely male and the second most-likely male. A large number of computer simulations (10,000) were used to find critical values of Δ for strict (95%) and relaxed (85%) confidence levels. In this study we assumed the absence of genotyping errors in the microsatellite analysis and that the probability of mutation between parents and offspring was negligible. Progenies that were not assigned to a specific known pollen parent were classified as generated from a *J. regia* male located outside the study area. To detect sampling errors, maternity was re-assigned by combining the exclusion method with the

maximum-likelihood approach (identifying one parent with no information about the other).

Two parental exclusion probabilities (EP1 and EP2) were also calculated for each locus and over all loci with CERVUS software. Exclusion probability second parent (EP2), was the probability that an unrelated male, randomly sampled, would be excluded as a pollen donor in a paternity analysis when the mother's genotype is known; whereas exclusion probability first parent (EP1) represented the probability that an unrelated tree, randomly sampled from the population, would be excluded as a parent of the offspring.

### Pollination pattern

The distance between the mother tree (black walnut) and all assigned fathers (Persian walnut) for each progeny was measured to determine whether male reproductive success was influenced by the spatial arrangement of the adults trees. A Kruskal–Wallis *H* nonparametric test was used to compare the mean pollination distances of the hybridogenic *J. nigra* mother plants from each *J. regia* tree. The Spearman rank correlation between the probability that a tree was a pollen donor and its distance from each *J. nigra* mother tree was determined using XLSTATS software.

## Results

### DNA fingerprinting analysis

The 600 total samples analyzed with ten SSRs generated 129 alleles, an average of 12.9 alleles per locus, ranging from nine alleles in WGA69 and WGA9, to 23 in WGA276 (Table 3). The Principal Coordinate Analysis performed on the Simple Match's similarity coefficient computed using 129 alleles (binary code) revealed distinct *J. nigra* and *J. regia* clusters and the presence of several intermediate individuals. Three main groups were detected (Fig. 1); two that included 49 *J. regia* and 82 *J. nigra* trees were clearly separated by the first principle coordinate, which accounted for 19.89% of the variance. The second principal coordinate, explaining 8.22% of the variance, divided the *J. nigra* trees in two subgroups. *J. nigra*-NC plants, located outside the Park, were found to be genetically distinct from the other eastern black walnut trees planted inside the Park. Seven diploid hybrids (H1, H2, H19, IMP3, IMP4, IMP9 IMP18) were incorporated in the third main group, located in an intermediate position between black and common walnut. As expected, the triploid hybrid plant (N21), with two genome parts of *J. nigra* and one part of *J. regia*, was placed between black walnut and the hybrid groups. Cluster analysis showed that the third group was composed of genotypes genetically distinct from individuals of the two parental species, but this placement does not prove the trees in this group are all interspecific hybrids. The identification of diploid hybrids was definitively performed by assigning 459 offspring genotypes to four putative classes, corresponding to two black walnut (*J. nigra* N, *J. nigra* NC), one *J.regia*, and one *J.* × *intermedia* group. The assignment tests, the Paetkau et al. (1995) frequency method and Rannala and Mountain (1997) partial Bayesian method, combined with the exclusion-simulation significance test of Cournet et al. (1999), assigned 198 progeny seedlings to *J.* × *intermedia* Carr. class and the remaining 261 offspring genotypes to *J. nigra* N with a high statistical level of confidence. A total of 205 diploid hybrid plants were identified among the germplasm collected in Villa Mezzalira Park. As described in detail below, two of the putative offspring hybrids displayed a triploid genotype at only one microsatellite locus and for this reason were excluded from the population assignment analysis.

The microsatellite loci revealed a high level of variability in the tested samples (Table 3). As expected, deviations from Hardy–Weinberg equilibrium (HWE) were significant in the *J.* × *intermedia* group, consistent with more frequent departures from random mating in the hybrid population. An heterozygote excess was detected for nine loci (average: $H_o = 0.887$, $H_E = 0.628$). No significant deviation from HWE was found in *J. nigra* NC and *J. regia* genotypes, and in the *J. nigra* N group only WGA89 showed significant deviation from Hardy Weinberg equilibrium. The probability that two unrelated individuals would share the same genotypes ($PI_{unb}$) and the probability that two full-sibs will have identical genotypes ($PI_{sib}$) were extremely low in most cases. Across all 10 loci, $PI_{unb}$ and $PI_{sib}$ values ranged from $2.3 \times 10^{-6}$ (*J. nigra* N) to $1.8 \times 10^{-10}$

**Table 3** Diversity parameters measured in the two subgroups of black walnut (*J. nigra* N and *J. nigra* NC)

| Source | Locus | $N_a$ | $N_e$ | $H_o$ | $H_E$ | Chi Sq. Prob[a] | Probability of identity | | Exclusion probability | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | $PI_{unb}$ | $PI_{sib}$ | EP1 | EP2 |
| *Juglans nigra* N ($N = 328$) | WGA1 | 9 | 2.236 | 0.561 | 0.553 | 1.000 $^{ns}$ | 0.277 | 0.543 | 0.160 | 0.286 |
| | WGA4 | 9 | 1.893 | 0.530 | 0.472 | 0.696 $^{ns}$ | 0.295 | 0.588 | 0.129 | 0.299 |
| | WGA9 | 8 | 2.586 | 0.640 | 0.613 | 0.336 $^{ns}$ | 0.206 | 0.495 | 0.206 | 0.363 |
| | WGA69 | 5 | 1.368 | 0.299 | 0.269 | 0.430 $^{ns}$ | 0.545 | 0.752 | 0.038 | 0.147 |
| | WGA89 | 10 | 3.684 | 0.796 | 0.729 | 0.010* | 0.118 | 0.415 | 0.320 | 0.495 |
| | WGA118 | 8 | 2.460 | 0.674 | 0.594 | 0.110 $^{ns}$ | 0.230 | 0.511 | 0.191 | 0.336 |
| | WGA202 | 8 | 1.387 | 0.287 | 0.279 | 0.940 $^{ns}$ | 0.528 | 0.743 | 0.042 | 0.158 |
| | WGA276 | 10 | 2.326 | 0.573 | 0.570 | 0.203 $^{ns}$ | 0.215 | 0.519 | 0.188 | 0.364 |
| | WGA321 | 8 | 1.573 | 0.412 | 0.364 | 0.781 $^{ns}$ | 0.420 | 0.673 | 0.072 | 0.210 |
| | WGA331 | 8 | 2.787 | 0.662 | 0.641 | 0.990 $^{ns}$ | 0.191 | 0.477 | 0.230 | 0.383 |
| | Average | 8.3 | 2.23 | 0.543 | 0.508 | | | | | |
| | Total | 83 | | | | | $2.3 \times 10^{-6}$ | 0.003 | 0.828 | 0.976 |
| *Juglans nigra* NC ($N = 15$) | WGA1 | 4 | 1.891 | 0.533 | 0.471 | 0.955 $^{ns}$ | 0.318 | 0.594 | 0.116 | 0.262 |
| | WGA4 | 6 | 4.891 | 0.800 | 0.796 | 0.397 $^{ns}$ | 0.071 | 0.370 | 0.421 | 0.600 |
| | WGA9 | 6 | 3.435 | 0.800 | 0.709 | 0.685 $^{ns}$ | 0.120 | 0.426 | 0.310 | 0.492 |
| | WGA69 | 4 | 2.332 | 0.467 | 0.571 | 0.226 $^{ns}$ | 0.236 | 0.523 | 0.171 | 0.327 |
| | WGA89 | 7 | 3.689 | 0.800 | 0.729 | 0.224 $^{ns}$ | 0.113 | 0.414 | 0.332 | 0.508 |
| | WGA118 | 7 | 2.744 | 0.667 | 0.636 | 0.347 $^{ns}$ | 0.162 | 0.473 | 0.245 | 0.429 |
| | WGA202 | 6 | 5.625 | 0.800 | 0.822 | 0.061 $^{ns}$ | 0.057 | 0.353 | 0.466 | 0.641 |
| | WGA276 | 8 | 5.172 | 0.600 | 0.807 | 0.056 $^{ns}$ | 0.060 | 0.362 | 0.455 | 0.632 |
| | WGA321 | 6 | 5.357 | 1.000 | 0.813 | 0.152 $^{ns}$ | 0.062 | 0.359 | 0.450 | 0.627 |
| | WGA331 | 7 | 4.787 | 0.800 | 0.791 | 0.693 $^{ns}$ | 0.073 | 0.373 | 0.418 | 0.596 |
| | Average | 6.1 | 3.992 | 0.72 | 0.714 | | | | | |
| | Total | 61 | | | | | $1.8 \times 10^{-10}$ | $1 \times 10^{-4}$ | 0.986 | 0.999 |
| *Juglans regia* ($N = 49$) | WGA1 | 4 | 2.853 | 0.612 | 0.650 | 0.548 $^{ns}$ | 0.196 | 0.474 | 0.215 | 0.361 |
| | WGA4 | 3 | 1.782 | 0.429 | 0.439 | 0.917 $^{ns}$ | 0.403 | 0.631 | 0.096 | 0.181 |
| | WGA9 | 3 | 2.441 | 0.531 | 0.590 | 0.391 $^{ns}$ | 0.241 | 0.515 | 0.174 | 0.312 |
| | WGA69 | 5 | 3.435 | 0.816 | 0.709 | 0.265 $^{ns}$ | 0.134 | 0.429 | 0.288 | 0.461 |
| | WGA89 | 3 | 2.823 | 0.653 | 0.646 | 0.993 $^{ns}$ | 0.198 | 0.477 | 0.209 | 0.355 |
| | WGA118 | 4 | 3.120 | 0.776 | 0.680 | 0.155 $^{ns}$ | 0.162 | 0.451 | 0.255 | 0.417 |
| | WGA202 | 5 | 3.482 | 0.735 | 0.713 | 0.639 $^{ns}$ | 0.131 | 0.426 | 0.295 | 0.467 |

**Table 3** continued

| Source | Locus | $N_a$ | $N_e$ | $H_o$ | $H_E$ | Chi Sq. Prob[a] | Probability of identity | | Exclusion probability | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | $PI_{unb}$ | $PI_{sib}$ | EP1 | EP2 |
| | WGA276 | 10 | 3.161 | 0.531 | 0.684 | 0.975 $^{ns}$ | 0.154 | 0.447 | 0.273 | 0.437 |
| | WGA321 | 5 | 3.349 | 0.633 | 0.701 | 0.983 $^{ns}$ | 0.148 | 0.436 | 0.270 | 0.434 |
| | WGA331 | 2 | 1.979 | 0.571 | 0.495 | 0.278 $^{ns}$ | 0.378 | 0.597 | 0.122 | 0.186 |
| | Average | 4.4 | 2.843 | 0.629 | 0.631 | | | | | |
| | Total | 44 | | | | | $9.3 \times 10^{-8}$ | $5 \times 10^{-4}$ | 0.919 | 0.989 |
| *Hybrid 2n* ($N = 205$) | WGA1 | 7 | 4.882 | 1.000 | 0.795 | 0.0** | 0.074 | 0.371 | 0.413 | 0.592 |
| | WGA4 | 5 | 2.531 | 1.000 | 0.605 | 0.0** | 0.235 | 0.506 | 0.188 | 0.322 |
| | WGA9 | 5 | 2.578 | 0.873 | 0.612 | 0.0** | 0.212 | 0.497 | 0.199 | 0.351 |
| | WGA69 | 1 | 1.000 | 0.000 | 0.000 | – | 1.000 | 1.000 | 0.00 | 0.00 |
| | WGA89 | 5 | 3.334 | 1.000 | 0.700 | 0.0** | 0.133 | 0.433 | 0.289 | 0.467 |
| | WGA118 | 6 | 4.306 | 1.000 | 0.768 | 0.0** | 0.094 | 0.390 | 0.362 | 0.540 |
| | WGA202 | 7 | 3.262 | 1.000 | 0.693 | 0.0** | 0.132 | 0.436 | 0.290 | 0.470 |
| | WGA276 | 15 | 5.095 | 1.000 | 0.804 | 0.0** | 0.067 | 0.365 | 0.438 | 0.614 |
| | WGA321 | 7 | 3.026 | 1.000 | 0.670 | 0.0** | 0.154 | 0.454 | 0.256 | 0.431 |
| | WGA331 | 4 | 2.720 | 1.000 | 0.632 | 0.0** | 0.204 | 0.485 | 0.204 | 0.353 |
| | Average | 6.2 | 3.273 | 0.887 | 0.628 | | | | | |
| | Total | 62 | | | | | $1.3 \times 10^{-8}$ | $7 \times 10^{-4}$ | 0.959 | 0.996 |

*J. regia* and their diploid hybrids: observed ($N_o$) and effective number ($N_e$) of alleles, observed ($H_o$) and expected heterozygosity ($H_E$), unbiased probability of identity ($PI_{unb}$), probability of identity between two random full sibs ($PI_{sib}$), exclusion probability (EP1 both parents unknown, EP2 maternal parent known)

[a] Level of significance of Chi square test (Hedrick 2000) for departures from Hardy–Weinberg : $^{ns}$ $P > 0.05$; ** $P < 0.001$

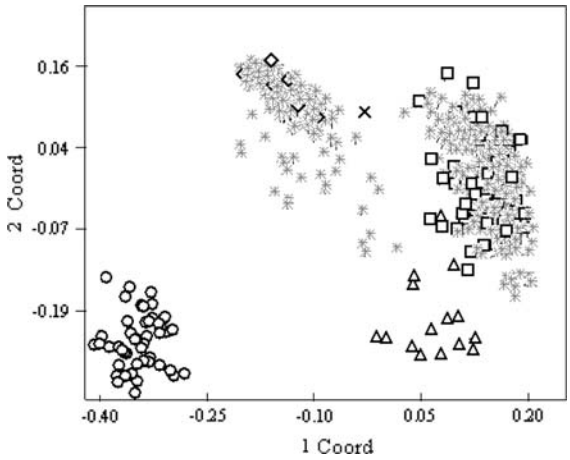


**Fig. 1** Principal Coordinate Analysis of 600 *Juglans* individuals based on genotypic similarity as determined by simple match coefficients based on 10 SSR loci. ○ *J. regia* ($N = 49$), □ *J. nigra* N ($N = 67$), △ *J. nigra* NC ($N = 15$), × N21 triploid hybrid, ◇ diploid hybrids ($N = 7$) and ✳ *J. nigra* offspring ($N = 461$)

(*J. nigra* NC) and from 0.003 (*J. nigra* N) to $7 \times 10^{-4}$ (*hybrid 2n*), respectively. The chance of finding two individuals with the same genotypes in each group was almost nil. Correspondingly, the probability that an unrelated male would be excluded as a sire in a paternity analysis where the maternal genotype is known (EP2), and the probability that an unrelated tree would be excluded as a parent of the offspring (EP1), was high for all populations. The combined power of exclusion, (probability considering all ten loci) ranged from 0.976 (*J. nigra* N) to 0.999 (*J. nigra* NC) for EP2 and from 0.828 (*J. nigra* N) to 0.986 (*J. nigra* NC) for EP1, indicating that the levels of polymorphism were sufficient for the subsequent parentage analysis beyond any reasonable doubt. Nevertheless, for locus WGA69, only one allele private to *J. nigra* was amplified in each hybrid tree. Thus, the presence of a null allele in *J. regia* was postulated. The null allele could be due to a mutation in the primer annealing sites (Pollegioni et al. 2009). Therefore, WGA69 which was useful for the maternity checks (*J. nigra* trees), was excluded from the parentage analysis because the occurrence of the null allele could cause mistakes in the paternity assignment (false exclusions of *J. regia* trees).

## Parentage analysis

A check of maternity based on simple exclusion using ten microsatellite loci was carried out on all 461 offspring divided into eight open-pollinated progenies (N3, N4, N17, N18, N21, N22, N23, N24). Progenies with alleles not found in the presumed maternal parent were considered to have resulted from accidental seed mixture. Results of maternity analysis in each half-sib family (Table 4) showed that mother-offspring mismatches occurred for only 12 seedlings, 9 in N18 and 3 in N22 progeny; no hybrid seedlings were involved in the above sampling errors. Maternity analysis of these offspring genotypes was resolved by the maximum-likelihood approach. Nine were reassigned to the half-sib family N17 and three

**Table 4** Maternity analysis and identification of the hybridogenic mother trees

| Maternal tree | Number of putative offspring | Non-maternity (seed mixture) | Maternity assignment[b] | Total number of offspring | Hybrid progeny *J.* × *intermedia* Carr (N)[c] |
|---|---|---|---|---|---|
| N3 | 41 | 0 | – | 41 | 0 |
| N4 | 29 | 0 | – | 29 | 0 |
| N17 | 88 | 0 | – | 97 | 68 (70%) |
| N18 | 24 | 9 | N17 (9) | 15 | 0 |
| N21 | 18 | 0 | – | 18 | 15 (83.3%)[a] |
| N22 | 76 | 3 | N23 (3) | 73 | 0 |
| N23 | 71 | 0 | – | 74 | 17 (22.9%) |
| N24 | 114 | 0 | – | 114 | 100 (87.7%) |

[a] Two hybrids offspring, N21-14 and N21-15, triploid for one locus, were included

[b] The maternity was re-assigned combining the exclusion method based on Mendelian segregation rules with maximum-likelihood approach (Marshall et al. 1998)

[c] Based on genotyping eight half-sib progenies using ten microsatellite loci

**Table 5** SSR genotypes for 18 seedling offspring of N21 (triploid hybrid): 13 diploid hybrid (*hyb 2n*), two hybrids triploid at one locus (*hyb 3n*), and three *J. nigra* genotypes

| SSR Locus | | WGA1 | | | WGA4 | | | WGA9 | | | WGA69 | | | WGA89 | | | WGA118 | | | WGA202 | | | WGA276 | | | WGA321 | | | WGA331 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 | A1 | A2 | A3 |
| N21 | Mother | 187 | 185 | 180 | 250 | 248 | 233 | 247 | 247 | 239 | 171 | 171 | * | 201 | 207 | 211 | 221 | 226 | 198 | 252 | 246 | 260 | 153 | 149 | 189 | 244 | 244 | 226 | 181 | 181 | 270 |
| N21-14 | *hyb 3n* | 187 | 180 | | 250 | 231 | | 239 | 247 | | 171 | * | | 201 | 221 | | 226 | 198 | | 252 | 267 | | 149 | 177 | 189 | 244 | 239 | | 181 | 274 | |
| N21-15 | *hyb 3n* | 185 | 192 | 180 | 248 | 233 | | 247 | 247 | | 171 | * | | 207 | 215 | | 226 | 183 | | 252 | 265 | | 149 | 191 | | 239 | - | | 181 | 270 | |
| N21-1 | *hyb 2n* | 185 | 192 | | 248 | 233 | | 239 | 247 | | 171 | * | | 201 | 211 | | 221 | 196 | | 252 | 267 | | 153 | 189 | | 244 | 226 | | 181 | 270 | |
| N21-2 | *hyb 2n* | 187 | 180 | | 248 | 233 | | 239 | 247 | | 171 | * | | 207 | 221 | | 221 | 198 | | 252 | 267 | | 153 | 189 | | 244 | 226 | | 181 | 270 | |
| N21-4 | *hyb 2n* | 187 | 190 | | 248 | 233 | | 239 | 247 | | 171 | * | | 201 | 215 | | 221 | 198 | | 246 | 275 | | 149 | 177 | | 244 | 226 | | 181 | 270 | |
| N21-5 | *hyb 2n* | 185 | 190 | | 250 | 233 | | 243 | 247 | | 171 | * | | 207 | 215 | | 226 | 196 | | 252 | 265 | | 149 | 189 | | 244 | 243 | | 181 | 270 | |
| N21-6 | *hyb 2n* | 185 | 190 | | 250 | 233 | | 239 | 247 | | 171 | * | | 207 | 215 | | 226 | 196 | | 246 | 275 | | 153 | 189 | | 244 | 243 | | 181 | 274 | |
| N21-7 | *hyb 2n* | 187 | 190 | | 250 | 233 | | 243 | 247 | | 171 | * | | 207 | 211 | | 221 | 196 | | 246 | 265 | | 149 | 177 | | 244 | 239 | | 181 | 270 | |
| N21-8 | *hyb 2n* | 187 | 192 | | 248 | 233 | | 239 | 247 | | 171 | * | | 201 | 215 | | 226 | 198 | | 246 | 275 | | 153 | 173 | | 244 | 226 | | 181 | 270 | |
| N21-9 | *hyb 2n* | 187 | 192 | | 250 | 231 | | 247 | 247 | | 171 | * | | 207 | 215 | | 226 | 183 | | 246 | 265 | | 153 | 189 | | 244 | 239 | | 181 | 270 | |
| N21-10 | *hyb 2n* | 187 | 180 | | 250 | 233 | | 239 | 247 | | 171 | * | | 207 | 211 | | 226 | 198 | | 252 | 275 | | 149 | 189 | | 244 | 243 | | 181 | 270 | |
| N21-11 | *hyb 2n* | 185 | 180 | | 250 | 233 | | 239 | 247 | | 171 | * | | 207 | 211 | | 221 | 198 | | 252 | 265 | | 149 | 189 | | 244 | 226 | | 181 | 270 | |
| N21-12 | *hyb 2n* | 187 | 180 | | 248 | 233 | | 247 | 247 | | 171 | * | | 201 | 221 | | 221 | 198 | | 246 | 265 | | 153 | 189 | | 244 | 239 | | 181 | 274 | |
| N21-13 | *hyb 2n* | 185 | 190 | | 250 | 231 | | 239 | 247 | | 171 | * | | 207 | 215 | | 226 | 196 | | 246 | 267 | | 149 | 177 | | 244 | 243 | | 181 | 270 | |
| N21-16 | *hyb 2n* | 185 | 190 | | 248 | 233 | | 239 | 247 | | 171 | * | | 201 | 221 | | 221 | 196 | | 246 | 265 | | 149 | 189 | | 244 | 239 | | 181 | 270 | |
| N21-3 | *J.nigra* | 187 | 187 | | 248 | 252 | | 228 | 247 | | 171 | 171 | | 207 | 190 | | 221 | 221 | | 252 | 260 | | 144 | 149 | | 246 | 244 | | 181 | 195 | |
| N21-17 | *J.nigra* | 187 | 185 | | 248 | 240 | | 255 | 247 | | 171 | 171 | | 207 | 201 | | 221 | 221 | | 246 | 256 | | 153 | 153 | | 244 | 244 | | 181 | 181 | |
| N21-20 | *J.nigra* | 185 | 185 | | 248 | 250 | | 228 | 247 | | 171 | 175 | | 207 | 190 | | 221 | 223 | | 252 | 252 | | 153 | 161 | | 244 | 244 | | 181 | 181 | |

A1 = Allele1; A2 = Allele2; A3 = Allele3

Allele length in base pairs (*bp*), * indicates a null allele. *Shading*: 50% gray for *J. nigra*-private alleles, 25% gray for *J. regia*-private alleles, unshaded cells for common alleles

*A1* Allele1, *A2* Allele2, *A3* Allele3

to N23 with 95% confidence. As indicated in Table 4, analysis of the progenies revealed the presence of four hybridogenic mother trees. One-hundred eighty-five out of the 198 diploid hybrid offspring were grown from seeds harvested from N17, N23 and N24 *J. nigra* trees. The proportion of seedlings from these tree mothers resulting from crosses with *J. regia* was 70% (68/97) for the family N17, 22.9% (17/74) for N23 and 87.7% (100/114) for N24 (Table 4). Finally, 15 out of 18 seedlings from the N21 (triploid hybrid tree) family were identified as *J.* × *intermedia*, including two atypical hybrid samples (Table 5). The genotypic profile of N21-14 and N21-15 plants showed one *J. nigra* and two *J. regia* private alleles at locus WGA276 and WGA1, respectively. In both cases, the most likely explanation is the presence of an unreduced gamete that inherited two maternal alleles, one *J. nigra* (185 bp and 149 bp for N21-15 and N21-14, respectively) and one *J. regia* private allele (180 and 189 bp for N21-15 and N21-14, respectively). In addition, for the primer pair WGA321, no *J. nigra* and only one *J. regia* private allele was amplified in plant N21-15. Unfortunately, because N21-14 and N21-15 died 6 months after germination, their chromosome number was not verified with a full cytological analysis.

Results of paternity assignment for 198 diploid hybrid seedlings in four half-sib families (N17, N21, N23, N24) showed there was no self-pollination in the samples (Table 6). All four hybridogenic mother trees were pollinated by a remarkably high numbers of pollinators located outside the study site. Ninety-five hybrid seedlings were produced by immigrant pollen, the remaining 103 plants had pollen donors within the research site. The proportion of hybrid seedlings resulting from crosses with *J. regia* trees growing inside the Villa Mezzalira's park was 58.83% for family N17, 61.5% for N21, 32.29% for N23, 49% for N21 (mean = 51.15%). The paternity of four offspring (three genotypes of N24 and one of N17) was left unassigned because two males were equally likely. Twenty-six (53%) out of the total 49 *J. regia* genotypes sampled in the research site were pollen donors (Fig. 2). Among identified pollen donors, only 10 (V5, V7, V15, B4, B6, B7, B12, B13, B17, B19) (20.0%) sired more than two seedlings; the maximum values were 22 offspring for B6, 17 for V15 and 10 for B7, over all females.

**Table 6** Paternity analysis of 198 diploid hybrid offspring identified in four half-sib families (N17, N21, N23, N24)

| Maternal tree (*J. nigra*) | Diploid hybrid offspring (N) | Offspring sired by *J. regia* outside study site (N) | Offspring sired by *J. regia* within study site (N) | Within-site (*J. regia*) pollen donors (N) | Most-likely fathers (cases)[b] | Mean distance (m)[a] |
|---|---|---|---|---|---|---|
| N17 | 68 | 28 (41.17%) | 40 (58.83%) | 16 | V3 (1), V4 (1), V5 (1), V7 (1), V15 (10), V16 (1), B3 (2), B4 (1), B6 (9), B7 (2), B8 (1), B10 (1), B12 (1), B13 (4), B17 (2), B19 (1) | 38.75 ± 6.96 |
| N21 | 13 | 5 (38.5%) | 8 (61.5%) | 8 | V1 (1), V5 (1), V7 (1), V15 (1), B5 (1), B6 (1), B12 (2) | 98.23 ± 3.96 |
| N23 | 17 | 11 (64.70%) | 6 (35.29%) | 6 | V17 (1), B4 (1), B14 (1), B17 (1), B19 (1), R10 (1) | 74.58 ± 12.23 |
| N24 | 100 | 51 (51%) | 49 (49%) | 18 | V3 (1), V4 (1), V5 (1), V7 (2), V11 (2), V12 (1), V15 (6), B3 (1), B4 (1), B6 (12), B7 (7), B13 (2), B14 (1), B17 (3), B19 (1), R7 (1), R9 (2), AE (1) | 46.98 ± 19.37 |

[a] Mean distance between the mother and the most-likely assigned father

[b] Paternity analysis based on most-likelihood approach (Marshall et al. 1998; CERVUS software)

A detailed analysis of each progeny showed the highest number of fertilizations from a single pollen donor was ten (male V15) for N17 (female), 12 (male B6) for N24 (female) and 2 (male B12) for N21 triploid hybrid female tree. No pollen donor sired more than one offspring among the 74 N23 progeny.

The mean distance of pollen donors from female partners varied among female trees; males sampled by tree N21 (98.23 ± 3.96 m) and N23 (74.58 ± 12.23 m) were farther away than the successful males for trees N17 (38.75 ± 6.969 m) and N24 (46.98 ± 19.m) (Table 5). These were highly significant differences (Kruskal–Wallis $H$ tes, $P = 3.8e^{-7}$). The distribution pattern of mating distance illustrated that 22 out of the 36 *J. regia* trees located in the eastern side of the park sired 96% of the seedlings (Fig. 3). We found no significant correlation between reproductive success of common walnut trees and distance from black walnut mother plants (N17, $r_s = 0.194$ ns; N21, $r_s = -0.612$ ns; N23, $r_s = 0$; N24, $r_s = -0.00384$ ns) using Spearman rank correlation.

## Discussion

SSR markers are characterized by hypervariability, abundance, high reproducibility, Mendelian inheritance, and co-dominant expression. These positive features make them suitable tools for parentage analysis (Streiff et al. 1999; Tabbener and Cottrell 2003). We found that the ten SSR primer pairs used to perform the DNA fingerprinting and parentage tests in this study efficiently discriminated among black and common walnut genotypes and their interspecific hybrids (*J. nigra* × *J. regia*) and detected hybridogenic *J. nigra* female trees that spontaneously cross with *J. regia* trees (hybridogenic males).

As reported for other species, the high levels of polymorphism, typical of microsatellite loci, positively influenced the exclusion and identity probabilities (Streiff et al. 1999; He and Smouse 2002; Chaix et al. 2003). The allelic richness and the observed heterozygosity measured for each locus in the tested samples provided high combined power of exclusion and low probability of identity, reflecting the correlation among these functions. Nevertheless, the range of PI (both $PI_{unb}$ and $PI_{sib}$) and EP (both EP1 and EP2) detected in this study were lower than the corresponding values reported for 39 open-pollinated families of *J. nigra* species collected in ten different American states (Robichaud et al. 2006). This result was not surprising, given the relative sizes of the respective populations evaluated; nevertheless, our study clearly demonstrated the power of the SSR markers we employed for DNA fingerprinting and parentage analysis.

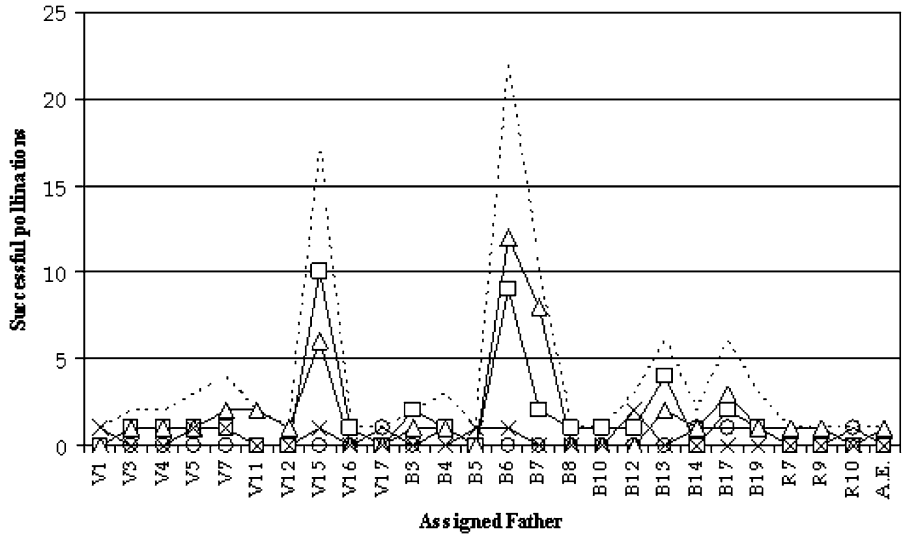


**Fig. 2** Number of hybrid offspring produced by each *J. regia* male that pollinated *J. nigra* females, -□- N17, -×- N21, -○- N23, -△- N24, and - - - - total. Assignment was based on greatest likelihood. The successful pollinations corresponded to the number of times a pollen donor (*J. regia*) pollinated a mother tree (*J. nigra*)

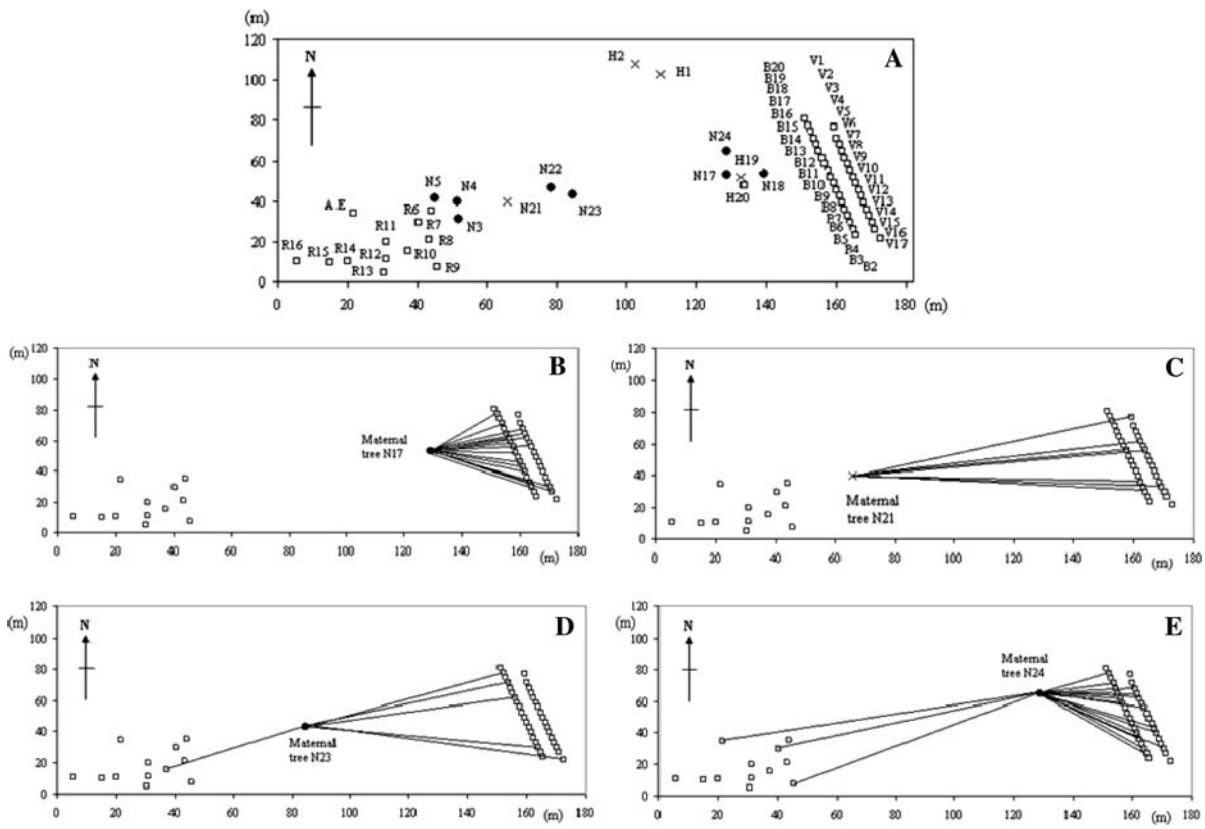


**Fig. 3** **a** Locations of the 61 adult walnut trees growing in the Villa Mezzalira Park: 8 *J. nigra* (●), 4 *J.* × *intermedia* Carr. (×) and 49 *J. regia* (□) plants. Geographic distribution of pollen donors for four hybridogenic mother trees, N17 (**b**), N21 (**c**), N23 (**d**), N24 (**e**) detected in Villa Mezzalira's Park

We identified 198 diploid *J.* × *intermedia* among 459 progeny. Maternity checks detected a small number of sampling errors (0.06%), probably resulting from accidental mixing of the seeds during the collection of the progenies. The identification of four (instead of only one) distinct hybridogenic *J. nigra* mother trees living in the Villa Mezzalira park was an important practical result. Indeed, the genetic improvement, especially of long living plants, requires the availability of selected "plus" genotypes able to produce a consistent quantity of hybrid progeny. Our approach also permitted the quantification of the differential reproductive success of each mother. Thus, even though these results should be confirmed by observations over additional years, we can already point to two plants with a relatively high rate of hybrid production: N24 (87%) and N17 (70%).

We found that the triploid hybrid plant N21 produced fertile female flowers, although the number of progeny was limited (18 total seedlings: 15 hybrid and 3 *J. nigra* genotypes). As described by Funk (1970) some *J.* × *intermedia* trees flower profusely but never bear much seed. In addition two hybrid plants out of 15 displayed a unusual and fatal karyotype: N21-14 and N21-15 plants showed one *J. nigra* and two *J. regia* private alleles at WGA276 and WGA1, respectively. Moreover, N21-15 revealed no *J. nigra* and only one *J. regia* private allele at locus WGA321. The most likely explanation for these microsatellite profiles is irregular meiosis in the original triploid hybrid and subsequent elimination/addition of chromosomes.

In this study we demonstrated the use of microsatellites to quickly identify male parents that spontaneously and frequently contribute to hybrids. Paternity of 198 diploid hybrids in four open-pollinated families was inferred by using a maximum likelihood approach (Marshall et al. 1998) based on nine microsatellite loci. We found that a considerable proportion of the hybrid offspring (mean value 51.15%) was sired by common walnut trees growing outside the research site. Clearly the research site was not spatially isolated from other populations, and there was a high proportion of pollen flow into the study site. Heavy pollen contamination, ranging from 21.6 to 63%, has been observed in a number of studies of wind- and insect-pollinated species, including *Eucalyptus grandis* (Chaix et al. 2003), *Quercus mcrocarpa* (Dow and Asley 1998), *Fagus crenata* (Asuka et al. 2005), *Magnolia obovata* (Isagi et al. 2004) and *Juglans mandshurica* (Bai et al. 2007). The large proportion of pollen donors from outside the stand confirmed that common walnut pollen regularly moves long distances, as far as mile, although effective natural pollination is generally limited to 60–100 m according to Griggs (1953). As reported by Impiumi and Ramina (1967), when wind speed was low (0.5 m/s), common walnut pollen showed uniform density for 160 m surrounding a source. In our study the mean distance of pollen donors within the site ranged from about 39–98 m, and the immigrant pollen traveled at least 110 m.

Differential male reproductive success was observed among pollen donors within the research site. In the production of hybrid progeny, male reproductive success was unevenly distributed both in genotypes and in space. *J. regia* trees located near the eastern edge of the study site were more successful at fertilizing *J. nigra* hybridogenic genotypes growing in the center of the stand than those on the western edge. Ten of the 36 common walnut trees at the eastern side of the park were effective pollinators, siring more than two hybrid offspring each, whereas 14 individuals provided no male gametes to hybrid progenies at all. In particular, 49 (47.5%) of the diploid hybrids with fully assigned parentage detected in four half-sib families were sired by three *J. regia* genotypes only (B6, V15 and B7). Uneven male contribution has been demonstrated in many plants (e.g., He and Smouse 2002; Chaix et al. 2003; Isagi et al. 2004; Grattapaglia et al. 2004). Although we did not record phenological data for the individuals in the research site, and our experimental design could not differentiate among all possible reasons for unequal paternal success, the results do guide speculation. *Juglans nigra* generally blooms later than *J. regia*, so the amount and timing of pollen shed, distance of pollen donor from seed trees, plant size, and weather conditions, may have had a profound effect on the distribution of male reproductive success (Asuka et al. 2005; Wheeler et al. 2006). We did not find a significant correlation between reproductive success of common walnut trees and distance from black walnut mother plants. Spatial factors may have influenced pollination in our study, but they were probably not a major determinant of male success. The timing of pollen release and the presence of some mechanisms of genetic incompatibility could be plausible explanations for the observed fertilization

pattern. The paternal plants may have been the only trees releasing pollen when the maternal trees had receptive stigma (synchronous flowering). On the other hand, pre-zygotic factors, such as pollen germination and tube growth rate, or post-zygotic factors, such as genetic complementation, could have affected male reproductive rate and may have been particularly relevant in this case where inter-specific crosses were made (Wheeler et al. 2006).

Although fluctuations in pollen production can occur among years, and the experiment was carried out on a relatively small sample of parent trees, parentage analysis of half-sib families based on microsatellite markers permitted the identification of new interspecific hybrids and, at the same time provided a rough idea of which walnut genotypes might be useful for establishing new seed orchards for inter-specific $F_1$ hybrid production. The use of genotypes with demonstrated compatibility may increase the efficiency of $F_1$ production. This method should also provide a powerful tool to evaluate the barriers to hybridization between *Juglans* species and to detect the factors that reduce hybrid fertility. Finally the retrospective selection of hybridogenic trees is a valid approach for the identification of new parental combinations when no phenological and morphological data of the trees are available.

In conclusion, in this study we successfully applied parentage analysis supported by microsatellite genotyping to identify hybridogenic mother trees and the most reproductively successful male parents.

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## References

Aitkin CGG (1995) Statistics and the evaluation of evidence for forensic scientists. Wiley, Chichester, UK

Asuka Y, Tomaru N, Munehara Y, Tani N, Tsumura Y, Yamamoto S (2005) Half-sib family structure of *Fagus crenata* saplings in an old-growth beech-dwarf bamboo forest. Mol Ecol 14:2565–2575. doi:10.1111/j.1365-294x.2005.02585.x

Bai WN, Zeng YF, Zhang DY (2007) Mating patterns and pollen dispersal in a heterodichogamous tree, *Juglans mandshurica* (Juglandaceae). New Phytol 176:699–707. doi:10.1111/j.1469-8137.2007.02202.x

Becquey J (1990) Quelques précisions sur les noyers hybrides. Forêt enteprise 69:15–19

Beineke WF, Masters CJ (1977) Controlling pollination in black walnut. In: Proceedings 10th Central States Forest Tree Improvement Conference. Purdue University, W Lafayette, pp 66–72

Chaix G, Gerber S, Razafimaharo V, Vigneron P, Verhaegen D, Hamon S (2003) Gene flow estimation with microsatellites in a Malagasy seed orchard of *Eucaliptus grandis*. Theor Appl Genet 107:705–712. doi:10.1007/s00122-003-1294-0

Cournet JM, Piry S, Luikart G, Estoup A, Soligna M (1999) New methods employing multilocus genotypes to select or exclude populations as origins of individuals. Genetics 153:1989–2000

Dow BD, Asley MV (1998) High levels of gene flow in bur oak revealed by paternity analysis using microsatellites. J Hered 89:62–70. doi:10.1093/jhered/89.1.62

Evett I, Weir B (1998) Interpreting DNA evidence. Sinauer Associates, Sunderland, MA

Fady B, Ducci F, Aleta N, Becquey J, Diaz Vazquez R, Fernandez Lopez F, Jay-Allemand C (2003) Walnut demonstrates strong genetic variability for adaptive and wood quality traits in a network of juvenile field tests across Europe. New For 25:211–225. doi:10.1023/A:1022939609548

Funk D (1970) Genetics of black walnut. USDA For Serv Res Paper (WO), vol ii, Washington, pp 1–13

Grattapaglia D, Ribeiro VJ, Rezende GDSP (2004) Retrospective selection of elite parent trees using paternity testing with microsatellite markers: an alternative short term breeding tactic for *Eucaliptus*. Theor Appl Genet 109:192–199. doi:10.1007/s00122-004-1617-9

Griggs WH (1953) Pollination requirements of fruits and nuts. Calif Agr Exp Sta Exp Serv Circ 424:35. http://scholar.google.it/Griggs+WH+Pollination+requirements+of+fruits+and+nuts

He T, Smouse PE (2002) Paternity analysis in *Ophiopogon xylorrhizus* Wang et Tai (Liliaceae sl); selfing assures reproductive success. J Evol Biol 15:487–494. doi:10.1046/j.1420-9101.2002.00393.x

Hedrick PW (2000) Genetics of populations, 2nd edn. Jones and Bartlett, Boston

Impiumi G, Ramina A (1967) Ricerche sulla biologia fiorale e di fruttificazione del noce (*J regia*) I Osservazioni sulla morfologia fiorale e sul trasporto del polline. Rivista dell'Ortofrutticoltura italiana 51:538–543

Isagi Y, Kanazashi T, Suzuki W, Tanaka H, Abe T (2004) Highly variable pollination patterns in *Magnolia obovata* revealed by microsatellite paternity analysis. Int J Plant Sci 165:1047–1053. doi:10.1086/423883

Jay-Allemand C, Dufour J, Germain E (1990) Detection précoce et rapide des noyers hybrids interspécifiques (*Juglans nigra* × *Juglans regia*) au moyen de critères morphologiques. PHM-Revue Horticole 311:39–41

Luza JG, Polito VS (1985) In vitro germination and storage of English walnut pollen. Sci Horti 27:303–316. doi: 10.1016/0304-4238(85)90035-4

Luza JG, Polito VS (1988a) Microsporogenesis and anther differentiation in *Juglans regia* L, a developmental basis for heterodichogamy in walnut. Bot Gaz 149:30–36. doi: 10.1086/337688

Luza JG, Polito VS (1988b) Cryopreservation of English walnut (*Juglans regia* L) pollen. Euphytica 37:141–148. doi:10.1007/BF00036851

Luza JG, Polito VS, Weinbaum SA (1987) Staminate bloom date and temperature responses of pollen germination and tube growth in two walnut (*Juglans*) species. Am J Bot 74:1898–1903. doi:10.2307/2443973

Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statical confidence for likelihhod-based paternity inference in natural populations. Mol Ecol 7:639–655. doi: 10.1046/j.1365-294x.1998.00374.x

McGranahan G, Voyiatzis D, Catlin P, Polito V (1994) High pollen loads can cause pistillate flower abscission in walnut. J Am Soc Hortic Sci 119:505–509

McKay JW (1965) Progress in black × Persian walnut breeding. Annu Rep North Nut Growers Assoc 56:76–80

McKay JW, McKay HH (1941) Microsporogenesis in *Juglans intermedia* Carr. Am J Bot 28:4s

Meagher TR (1986) Analysis of paternity within a natural population of *Chamaelirium luteum*. I. Identification of the most likely parents. Am Nat 128:199–215, Theor Popul Biol 29:87–106

Paetkau D, Calvert W, Stirling W, Strobeck C (1995) Microsatellite analysis of populatin structure in Canadian polar bears. Mol Ecol 4:347–354. doi:10.1111/j.1365-294X.1995.tb00227.x

Paetkau D, Waits LP, Clarkson PL, Craighead L, Vyse E, Ward R, Strobeck C (1998) Variation in genetic diversity across the range of North America brown bears. Conserv Biol 12:418–429. doi:10.1046/j.1523-1739.1998.96457.x

Paetkau D, Slade R, Burden M, Estoup A (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. Mol Ecol 13:55–65. doi:10.1046/j.1365-294X.2004.02008.x

Peakall R, Smouse PE (2005) GenAlEx V6: Genetic analysis in Excel population genetic software for teaching and research. The Australian National University, Canberra. http://wwwanueduau/BoZo/genAlEx/

Pollegioni P, Woeste K, Major A, Scarascia Mugnozza G, Malvolti ME (2009) Characterization of *Juglans nigra* (L), *Juglans regia* (L) and *Juglans x intermedia* (Carr) by SSR markers: a case study in Italy. Silvae Genet 58:68–78

Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. Proc Natl Acad Sci USA 94:9197–9201. doi:10.1073/pnas.94.17.9197

Robichaud RL, Glaubitz JC, Rhodes OE Jr, Woeste K (2006) A robust set of black walnut microsatellites for parentage and clonal identification. New For 32:179–196. doi: 10.1007/s11056-005-5961-7

Rohlf FJ (2001) NTSYS-pc numerical taxonomy and multivariate analysis system version 2.1. Exeter Software, East Setauket

Sartorius R (1990) Anatomische histologische und cytologische Untersuchungen zur Samenentwicklung bei der walnut (*Juglans regia* L) unter besonderer Berücksichtigung der apomoxis. Dissertation, Fakulät III, Agrarwissenschaften I der Universität Hohenheim, p 123

Scheeder T (1990) Juglans intermedia in einem Bestand am Kaiserstuhl AFZ Der Wald 45:1236–1237

Schreiner EJ (1960) Objectives of pest-resistance improvement in forest trees and their possible attainment. Proc 5th World Forestry Cong, Seattle, USA 2:721–727

Sokal RR, Sneath PHA (1963) Principles of numerical taxonomy. Freeman, San Francisco

Streiff R, Ducousso A, Lexer C, Steinkellner H, Gloessl J, Kremer A (1999) Pollen dispersal inferred from paternity analysis in a mixed oak stand of *Quercus robur* L and *Q petraea* (Matt) Liebl. Mol Ecol 8:831–841. doi:10.1046/j.1365-294X.1999.00637.x

Tabbener HE, Cottrell JE (2003) The use of PCR based DNA markers to study the paternity of poplar seedlings. For Ecol Manage 179:363–376. doi:10.1016/S0378-1127(02)00538-8

Wheeler N, Payne P, Hipkins V, Saich R, Kenny S, Tuskan G (2006) Polymix breeding with paternity analysis in *Populus*: a test for differentiation reproductive success (DRS) among pollen donors. Tree Genet Genomes 2:56–60. doi: 10.1007/s11295-005-0033-x

Zobel B, Talbert J (2003) Applied forest tree improvement. Blackburn Press, Caldwell, 505 p