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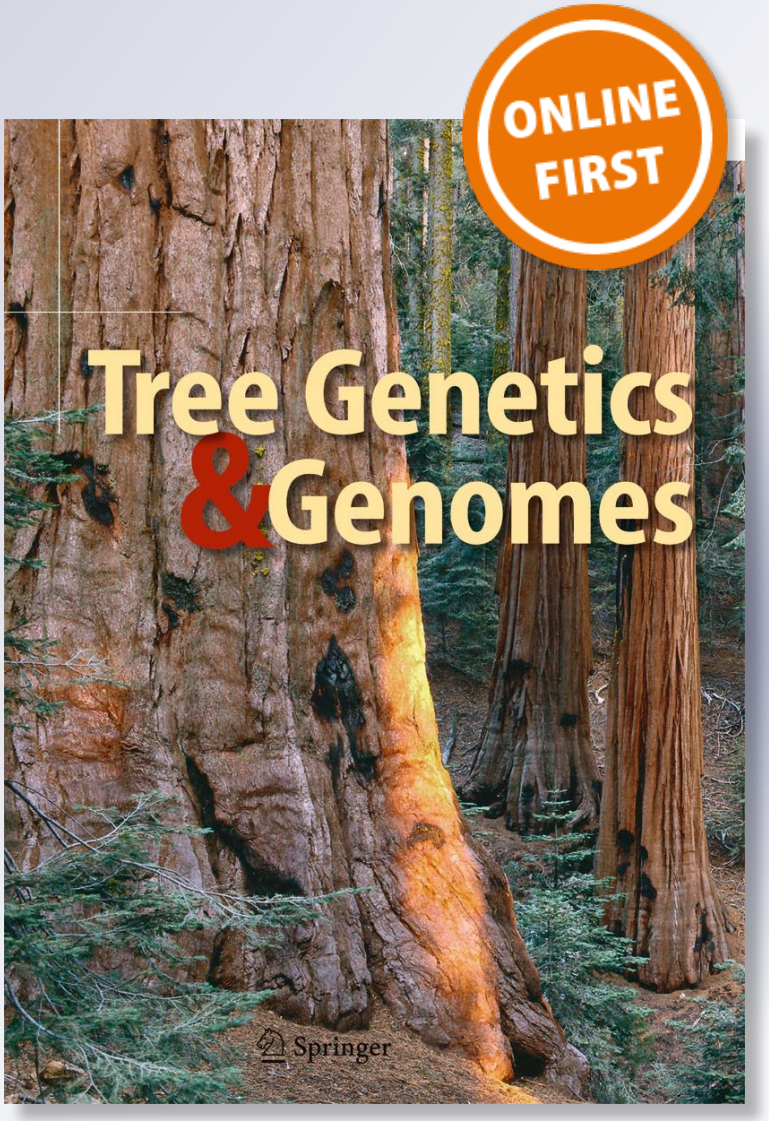

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ORIGINAL PAPER

Barriers to interspecific hybridization between *Juglans nigra* L. and *J. regia* L species

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Abstract *Juglans nigra* and *Juglans regia* are phylogenetically divergent species. Despite the economic interest in *Juglans* × *intermedia* (*J. nigra* × *J. regia*), walnut hybridization is rare under natural conditions and still difficult using controlled pollination. Here, we evaluated some reproductive mechanisms that may prevent successful natural hybridization. The study of flowering phenology of 11 *J. nigra* and 50 *J. regia* trees growing in a plantation provided information regarding the opportunity for interspecific crosses. Variation in flower size, pollen quality of putative donors, and variation in seed yield and rate of hybrid production among putative maternal trees were examined. DNA fingerprinting and parentage analyses based on nine microsatellites permitted the identification of hybrids and hybridogenic parent. Our data indicated that overlap occurred between the staminate flowering of protogynous *J. regia* and the beginning of pistillate flowering of protogynous *J. nigra*. Differences in floral size were computed between walnut species. Only three hybrids among 422 offspring of eleven *J. nigra* progenies were identified. Interspecific hybridization involving pollination of one early-flowering-protogynous *J. nigra* by three protogynous *J. regia* trees was detected. The correct development of *J. regia* male gametophytes, high pollen viability (86.5 %), and germination (57.6 %) ruled out the possibility that low pollen quality contributed to depressed hybrid production. Our findings indicated that these two species tended to remain reproductively isolated. The substantial disjunction in flowering time and additional prezygotic barriers such as differences in floral size and conspecific pollen advance may affect interspecific gene flow between *J. regia* and *J. nigra*.

Keywords Walnut hybridization · SSR fingerprinting · Prezygotic barriers · Bloom synchrony · Conspecific pollen advance · Floral differences

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Introduction

Interspecific hybridization between plant species is a common natural phenomenon of evolutionary importance (Lexer et al. 2004). Hybridization between native and non-native species can negatively affect biodiversity through several mechanisms including a decline in reproductive fitness and loss of genetic distinctiveness of native taxon by introgression (Wolf et al. 2001). On the other hand, interspecific hybridization played an essential role in allopolyploid and homoploid speciation in plant (Rieseberg 1997). Numerous studies systematically reported the presence of a small fraction of first-generation hybrid genotypes that exceeded their parents in vegetative vigor (heterosis) and/or display an environment-related superiority (Rieserberg and Carney 1998; Potts and Dungey 2004). As reported by Field et al. (2008), despite the potential adverse effects, interspecific hybridization still remains a powerful

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tool to generate novel genetic combinations in plant breeding programs. Thus, there has been a long-standing interest in understanding mechanisms of reproductive isolation affecting hybridization success. Indeed, interspecific hybrids are valued not only for their novel combination of desirable traits but also for characterizing phylogenetic affinity and to understand reproductive barriers (prezygotic and post-zygotic) to hybridization. These concerns have been particularly relevant for a group of economically important *Juglans* species for which interspecific hybridization events are possible but uncommon, and hybrids are known to vary in their vegetative vigor and fertility (Dandekar et al. 2005).

The *Juglans* genus consists of 21 species traditionally divided into four distinct sections (Manning 1978): (1) *Dioscaryon* Dode consisting solely of the highly valuable commercial Persian walnut species (*Juglans regia* L.) which is native to Eurasia from the Balkans to southwest China and is widely cultivated throughout the temperate regions of the world for its high quality wood and edible nuts, (2) *Rhysocaryon* Dode including 16 North and South American *Juglans* species such as the valuable hardwood species *Juglans nigra* (black walnut), (3) *Cardiocaryon* Dode including three species all native to East Asia, *Juglans ailantifolia* Carr., *Juglans cathayensis* Dode, and *Juglans mandshurica* Mahim, and (4) *Trachycaryon* consisting only of the North American species *Juglans cinerea*. Many of these species are capable of hybridizing, but the hybridization rate seems to reflect the phylogenetic relationships, with intra-sectional crosses more successful than inter-sectional crosses (McGranahan and Leslie 2009). In general, the black walnuts (*Rhysocaryon*) are unable to hybridize with species of sections *Trachycaryon* and *Cardiocaryon* with the exception of *J. nigra* and *J. ailantifolia* (Woeste and Michler 2011). On the contrary, although molecular studies confirmed the traditional taxonomic classification and supported section *Dioscaryon* as the oldest and phylogenetically separated lineage within *Juglans* spp. (Aradhya et al. 2007), Persian walnut shows an unexpected ability to cross, at least to some extent, with members of the other three sections acting as a bridge species (McGranahan and Leslie 2009).

In particular, in the last two decades renewed interest from both industry and academic researchers in the use of *J.* × *intermedia* Carr. (*J. nigra* × *J. regia*) for rootstock and/or timber production has been observed in many parts of Europe and US (Clark and Hemery 2010; Woeste and Michler 2011). *J. nigra* and *J. regia* are two phylogenetically divergent walnut species. Nowadays, both of species are widely cultivated throughout the temperate regions of the world and exhibited a broad area of sympatry (Aradhya et al. 2007). They are wind-pollinated, monoecious, and dichogamous species with the same number of chromosomes ($2n=32$). In particular, the mating system of both species exhibits a phenotypic dimorphism defined as "heterodichogamy": if the male flowers shed their pollen before the pistillate flowers are receptive, the genotypes are classified as "protandrous"; whereas if the mature pollen is released after the period of the female flower receptivity, the genotypes are classified as "protogynous" (Funk 1970). Compared to the parental species, most *J. nigra* × *J. regia* hybrids show increased vegetative vigor, distinct disease resistances, good wood quality, strong apical dominance, and resistance to spring frost damage (Fady et al. 2003; Chifflot et al. 2006). They were superior to the parental species in growth at sites with medium to low-fertility soils and were moderately tolerant to flooding. Despite the increasing demand for *J.* × *intermedia* trees for forestry, their large-scale production by controlled crosses is impractical due to pistillate flower abscission caused by excess of pollen load (McGranahan et al. 1994) and low viability of stored walnut pollen (Luza and Polito 1985). As reported in numerous studies (review in Woeste and Michler 2011), artificial crosses have been made between *J. nigra* and *J. regia,* but unfortunately, the resulting seed set was always very low (~0.3 %). Actually, the production of new *J.* × *intermedia* genotypes depends mostly on successful natural hybridization, but the percentage of hybrid progeny in a mixed population is usually less than 10 % (Funk 1970). The only exception is the establishment of seed orchards for hybrid production in France. The oldest and best known French *J.* × *intermedia* (NG23 × RA) was obtained by the open pollination of the female *J. nigra* NG23 with four *J. regia* trees as male parents (Becquey 1990). As reported also by Pollegioni et al. (2009), a few selected plants showed a particular aptitude for producing hybrids in nature and are defined "hybridogenic" plants.

Taking into account all this information, we postulated the presence of several pre- and post-zygotic mechanisms preventing hybridization between black and Persian walnuts which are rarely overcome in nature. However, these reproductive barriers still remain largely unknown. In particular, mechanisms such as differences in flowering times, divergence of floral characters, and pollen competition (conspecific pollen preference) are considered the most important prezygotic barriers to gene exchange between tree species (Ortiz-Barrientos and Rieseberg 2006). According to Luza et al. (1987), *J. regia* began flowering about 3 weeks prior *J. nigra,* and partial overlap in bloom time between the parental species likely provides opportunities for interspecific pollen flow. Therefore, we addressed the following questions: will the quantification of flowering synchrony between black and Persian walnut trees assist in understanding the degree of their natural hybridization? Is flowering overlap a reliable indicator of hybridogenic ability of walnut plants? What is the influence of the *Juglans* mating system (heterodichogamy) on the fertilization success between *J. nigra* and *J. regia*? Can the presence of additional prezygotic barriers, such as differences in floral

morphology and conspecific pollen preference, affect the hybridization rate between flowering synchronous Persian and black walnut trees and reduce the fertility in term of seed production?

For these purposes, we set out to (1) describe the flowering phenology of *J. nigra* and *J. regia* adult plants located in a mixed walnut plantation in Central Italy, clarifying the temporal patterns of the mating types within each species and quantifying the overlap of flowering times between walnut trees; (2) according to the short-term marker-assisted breeding tactic successfully used in *Juglans* by Pollegioni et al. (2009), detect the presence of first-generation *J.* × *intermedia* genotypes in eleven open-pollinated progeny arrays by analysis of SSR fingerprints (Bayesian approach) and identify hybridogenic walnut plants by parentage analysis; (3) evaluate the importance of various prezygotic barriers such as flowering synchrony, pollen competition, and difference in floral traits on seed production/germination and hybrid frequency among progeny arrays.

Materials and methods

Study site

The present study was conducted in the experimental field of the CNR-Institute of Agro-environmental and Forest Biology of Porano, (Biagio site, Terni, Italy, 1.2 ha, latitude 42°43′N, longitude 12°02′E) in a hilly volcanic region, 55 km east of the Mediterranean sea coast, at about 500 m a.s.l. This area is characterized by a transitional climate between the Mediterranean and cool sub-humid type with a mean annual rainfall of 827 mm and a mean annual temperature of 12 °C (Biagio weather station, 1992–2010). At this site, we selected a 19-year-old plantation made up of *J. regia* and *J. nigra*. Seedlings for the plantation were obtained from random seeds of *J. regia* and *J. nigra* provided by the Veneto regional nursery (Montecchio Precalcino, Vicenza, Italy) and French nursery (Lalanne, Bordeaux), respectively. In spring 2010, a total of 61 adult plants (11 *J. nigra* and 50 *J. regia*) were mapped and evaluated for their hybridization ability. The genotype identity (ID) of each walnut tree was reported in Online Resource 1.

Floral data

Flowering phenology

The pattern of flowering phenology of 11 *J. nigra* and 50 *J. regia* adult trees was monitored during spring 2010 and 2011. The date of onset and termination of male and female flowering were annotated daily from 26 March to 21 June 2010 and from 1 April to 31 May 2011. Four female inflorescences and one branch with male catkins (the staminate flowers are small and densely grouped in catkins borne laterally on 1-year-old wood) at each cardinal point were tagged in each walnut tree and evaluated. Flowering duration, flowering peak, and flowering synchrony were studied in each species. The protogynous (PG) and protandrous (PA) mating types were also determined for each walnut adult tree.

The duration of flowering was estimated as the number of days on which the walnuts remained in bloom. In particular, the period of female flowering was determined by observing the orientation of the two stigmatic lobes of the tagged pistillate flowers. As reported by Luza and Polito (1988), pistillate flowers were considered receptive when the two stigmatic lobes were separated from one another to form a V-shape with an orientation of 45° angle to the longitudinal axis of the ovary. The onset of female flowering was equated with the Julian day on which the first receptive pistillate flowers appeared. Cessation coincided with the last Julian day on which 100 % of selected pistillate flowers were no longer receptive. Timing of male flowering was calculated starting from the date of pollen shedding to the date when the color of all tagged catkins changed from yellow-green to brown (Kimura et al., 2003). The flowering peak was the date when the maximum number of receptive female flowers or shedding catkins was registered. The floral synchrony was determined by using the method outlined by Augspurger (1983). The Index of floral overlap (X_{ik}) reflects the days in which an individual i overlaps with the rest of population of species k and is calculated through the following formula:

$$X_{ik} = (1/1 - n)(1/f_i) \sum e_{j \neq i}$$

where e_j is the number of days during which both individuals j and i flower synchronously with $j \neq i$; f_i is the number of days in which individual i is in flower and n is the number of individuals in surveyed population of species K. X_{ik} may vary between 0 and 1; when $X_{ik}=1.0$ perfect flowering synchrony occurs, whereas for $X_{ik}=0$, there is no overlap. It should be noted that this method does not consider the amplitude of flowering: all flowering dates are considered equivalent regardless of the number of flowers produced.

In this study, taking into consideration the flowering duration data and the potential gene flow between walnut species, we selected 11 *J. nigra* adult plants as putative seed parents and 50 *J. regia* adult plants as putative pollen donors for the production of *J.* × *intermedia*. Hence, during 2010 and 2011, the asynchrony index was calculated for each maternal *J. nigra* adult tree (sampled for seed) with respect to the *J. nigra* and *J. regia* populations. In addition, temperature records during flowering in 2010 and 2011 were obtained from the Biagio weather station to test the effect of temperature on flowering time of walnut plants.

Flower size measurements

During spring 2010, we randomly sampled 16 pistillate flowers in the receptive phase and 16 mature catkins releasing pollen from each *J. nigra* and *J. regia* adult tree in the Biagio plantation. Five floral attributes were measured: ovary size including equatorial (A) and polar (B) diameter, style length, stigma length, and catkin length.

Pollen development, viability, and germination tests in vitro

In order to verify the correct development of pollen in the putative *J. regia* male parents (putative hybridogenic fathers), staminate flower buds at successive development stages from six protogynous genotypes (RAB9_PG, RAB18_PG, RAB27_PG, RAB37_PG, RAB66_PG, RAB82_PG) were examined. Male catkins were sampled from 26 March to 21 June 2010. In particular, anthers were collected from proximal part of 10-mm-long, 13-mm-long, 20-mm-long, and mature (~75-mm-long) catkins of each selected Persian walnut. Anthers were dissected, squashed on a microscope slide, and the released pollen grains were stained in a drop of 1 % acetic orcein and 50 % glacial acetic acid solution and visualized by light microscope (NIKON JAPAN 104). Micrographs were taken using a PHOTO-BIO (PbI-International) digital camera. Therefore, microsporogenesis and pollen development were tested as previously described by Luza and Polito (1988) and Olimpieri et al. (2011). In addition, pollen viability was determined at maturity for each selected plant. Dark purple pollen grains with cytoplasm stained by acid orcein were classified as viable, while light red-pink and not-dyed grains were considered as semi-viable and non-viable pollen, respectively. Ten fields were sampled by microscope; then, the mean percentage of stained pollen grains per plant was computed over a sample of at least 200 grains.

Finally, fresh pollen was collected from five mature catkins of each of six *J. regia* individuals to estimate the pollen germination rate in vitro. Pollen germination tests were conducted using the hanging drop method with 20 % sucrose (Mert 2009). After incubation at 28 °C in the dark for 24 h, pollen germination was determined under the light microscope. A pollen grain was considered germinated when the length of pollen tube was equal to or exceeded pollen diameter. Ten fields per genotype were observed, and germination was quantified as the percentage of germinated pollen grains in a sample of at least 200 grains counted.

Seed sampling

In light of the observed male and female flowering duration and floral synchrony between Persian and black walnut genotypes, variation in seed and hybrid production among black walnut trees was examined. In October 2010, seeds produced by open pollination from each of the eleven adult *J. nigra* trees were collected at the Biagio site. A total of 1,148 seeds, consisting of 15 to 201 seeds from each sampled tree (Online Resource 2) were morphologically evaluated using the UPOV (1999) guidelines for distinctness and uniformity. Five measurements were made on the seed arrays: (1) seed weight, (2) seed length (polar diameter, H), (3) seed width (equatorial diameter, L), (4) seed thickness (equatorial diameter, E), and index of roundness (R) calculated as follows: $R=(E+L)/2H$. Subsequently, all collected seeds were stratified in a moist peat at 4 °C for 90 days and then individually sown in a 1 sand: 1 peat: 1 compost soil mixture in separate pots (50 mm wide, 100 mm deep) to detect the germination rate under glasshouse conditions for each open-pollinated progeny array. As reported in Online Resource 2, we obtained a total of 422 germinated seedlings which were partitioned into eleven open-pollinated progenies and genotyped using microsatellite markers.

Genetic analysis

During late spring 2011, young leaves were sampled from the 422 germinated seedling, 11 *J. nigra* and 50 *J. regia* adult walnut plants. A total of 483 samples were stored at −80 °C until use. For each sample, 100 mg of leaf tissue was homogenized in a 2-ml microcentrifuge tube containing a 5-mm steel bead cooled with liquid nitrogen using Mixer Mill 300 (Qiagen, Hilden, Germany). 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.

All samples were genotyped using nine unlinked microsatellite loci (WGA1, WGA4, WGA9, WGA89, WGA118, WGA202, WGA276, WGA321, WGA331) previously selected, sequenced, and used for genetic characterization of Persian, black walnut, and *J.* × *intermedia* trees in Italy (Pollegioni et al. 2009, 2011; Table 1). The PCR amplification and the visualization of amplified SSR alleles for each sample were carried out as described by Pollegioni et al. (2009). 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 Biosystems, Foster City, CA, USA).

Data analysis

One-way analysis of variance (ANOVA) followed by post hoc Tukey's test (pairwise genotype comparisons) was conducted to detect significant differences in the flowering peak between protogynous and protandrous black and Persian walnut adult trees and compare their floral attributes (ovary size, style, stigma, and catkin length). The Chi-square test

Table 1 List of nine microsatellite markers used for genetic characterization of *J. nigra*, *J. regia,* and first-generation *J.×intermedia* Carr. trees in Italy (Pollegioni et al. 2009, 2011) (for each SSR, the expected allelic range (bp), expected species-private alleles (bp), expected common alleles (bp), and total number of alleles amplified on the 61 walnut adult trees and 422 seedling offspring obtained from the experimental plantation at the Biagio site are shown)

SSR locus	Expected allelic range (bp)	Expected species-private alleles (bp)		Expected common alleles (bp)	Total number of amplified alleles
		J. nigra	*J. regia*		
WGA1	179–211	179–181–183–185–187 196 198–200–211	180–188–192–194	189–190	6
WGA4	231–257	240–242–246–248–250 252 254–257	231–235	233–237	7
WGA9	228–261	228–241–251–253–255 257 261	239	243–247	6
WGA89	186–234	186–190–196–201–203–207 213–219–222–226 230–234	215–221	211	9
WGA118	183–244	208–210–212–214–219–221 223–226–228–236–242–244	183–196–198–206	–	9
WGA202	246–295	246–248-250–252–254 256 258	265–267–275–295	260	10
WGA276	144–195	144–147–149–153–155 157 159–161–163–165–167–169	171–173–175–177–179 181–183 187–189–191 195	–	13
WGA321	226–195	231–236–237–238–242–244 246–248–252–254–260–264	226–228–230–239 241–243 245	–	11
WGA331	177–274	177–179–181–183–185 187–189–191–195–199	270–274	–	6
Total					77

was performed to evaluate whether ratio of protogynous trees differed from that of protandrous trees within each species. The Student's*t* test was performed to detect variation in floral morphology between *J. nigra* and *J. regia* tree groups. ANOVA and post hoc Tukey's test were also used to determine differences in the mean morphological traits (weight, length, width, thickness) of seed arrays produced by open pollination from each of the eleven adult *J. nigra* trees. We examined the relationship between seed production characteristics and the degree of temporal synchrony of *J. nigra*-female flowering with *J. regia*−(X_{regia}) and *J. nigra* −(X_{nigra}) male flowering calculated for each *J. nigra* maternal tree during 2010. The Spearman rank correlation was calculated between floral synchrony index values, seed number, weight, length (*H*), width (*L*), thickness (E), shape index, percentage of germination, and hybrid production rate of seeds computed in eleven open-pollination progenies. All computations were performed by XLSTAT2011 software (http://www.xlstat.com).

Microsatellite diversity

Standard genetic diversity parameters were assessed separately for *J. nigra* and *J. regia* genotypes classified using Bayesian analyses (see below). Number of alleles per locus (Na), effective number of alleles (Ne), and observed (Ho) and expected (H_E) heterozygosity were calculated at each locus, and overall loci for all adult trees at the Biagio site and the offspring assigned to one of two classes. Departures from Hardy–Weinberg expectations at each locus were tested by a Chi-square test. 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. 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}). All calculations were performed using GenAlEx version 6 software (Peakall and Smouse 2005).

Hybrid identification

The Band–Sharing (BS) coefficient based on nine SSR markers (Lynch 1990) was computed between all pairwise combinations of individuals in order to evaluate the genetic relationships exiting between genotypes. We applied Principal Coordinate Analysis based on the BS matrix to display the relative genetic distances among the 483 genotypes (61 adult trees, 422 seedlings). Subsequently, a Bayesian model-based clustering method implemented in STRUCTURE software version 2.3.3 (Pritchard et al. 2000) was used to assign walnut genotypes to *J. nigra* and *J. regia* species and identify hybrid seedlings. This method attempts to assign individuals to K genetic clusters in order to minimize within-group linkage disequilibrium and deviation from Hardy–Weinberg equilibrium, computing the admixture coefficient or proportion of membership (*Q* value) for each genotype. STRUCTURE analysis was performed using the admixture model on the whole data set with no previous population information, the correlated allele frequencies between populations, and a burn-

in period of 10,000 steps followed by 10^5 MCMC replicates options. First, we conducted six independent runs at each K from 1 to 5 to determine the most likely number of clusters according to post hoc statistics of Evanno et al. (2005). Based on the initial results, we assumed $K=2$ as most likely number of clusters in agreement with the presence of two species (Q_1=*J. regia*, Q_2=*J. nigra*). Therefore, as suggested by Field et al. (2011), we classified individuals with $Q_1>0.9$ as *J. regia*, $Q_1<0.1$ (or $Q_2>0.9$) as *J. nigra* and $0.1\leq Q_1\leq 0.9$ as *J.×intermedia* hybrids. In order to perform a more detailed analysis of admixture proportions and confirm the previous hybrid assignment, the model-based Bayesian statistical approach implemented in NewHybrids software version 1.1 (Anderson and Thompson 2002) was applied. This method provides the posterior probability (q_i) of each individual belonging to six genotype frequency classes: (I) Purebred_*J. regia*, (II) Purebred_*J. nigra*, (III) F1 hybrid, (IV) F2 hybrid, (V) Bx *J. regia* (backcross towards *J. regia*), and (VI) Bx *J. nigra* (backcross towards *J. nigra*). As recommended, tests were carried out with a burn-in period of 20,000 generations and 200,000 MCMC replicates, with no previous population information and Jeffery's like priors parameters. In this study, posterior probabilities of 60 % (relaxed level) and 90 % (strict level) were considered as a statistical threshold for assignment analysis. In addition, to assess the efficiency of hybrid identification, we also included 384 *J. nigra*, 80 *J. regia,* and 205 first-generation *J.×intermedia* trees already genotyped with nine SSR markers (Pollegioni et al. 2009, 2011). These samples have been used as reference sets for PcoordA, STRUCTURE, and NewHybrids elaborations.

Assignment of parentage

Paternity analysis for each hybrid offspring was carried out using CERVUS ver. 2.0 (Marshall et al. 1998), a software based on the maximum-likelihood method. Paternity analysis (the assignment of a *J. regia* father to previously determined *J. nigra* mother−*J.×intermedia* offspring pair) was conducted on hybrid offspring with all *J. regia* reproductive trees at the site acting as potential fathers. Paternity was assigned to the male with the highest log-likelihood ratio (LOD score) among non-excluded males. Marshall et al. (1998) defined a $\Delta-$ statistic to assess the statistical confidence in the paternities that were assigned. In this approach, Δ value corresponded to the difference in LOD scores between the most likely male and the second most likely male. A number of 10,000 simulations were carried out 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. We also did not allow mismatching for any of the nine SSR loci. Two parental exclusion probabilities (EP1 and EP2) were also calculated for each locus and overall loci with CERVUS software.

Results

Flowering phenology, duration, and synchrony of walnut adult trees

A total of 11 *J. nigra* and 50 *J. regia* adult trees were monitored during the 2010 and 2011 bloom period at the Biagio experimental plantation. The flowering pattern of female and male inflorescences for each walnut plant is reported in Fig. 1. As expected, the mating types protogyny and protandry were observed during both study years and the correspondent mating type classification of individuals was consistent from year to year. A total of five protogynous (PG) and six protandrous (PA) *J. nigra* trees were detected with a ratio not significantly different from 1:1 ($\chi^2=0.09$, df=1, $P>0.05$). However, a protogynous-biased ratio was observed in *J. regia* ($\chi^2=3.92$, df=1, $P<0.05$), including 32 protogynous and 18 protandrous plants (Online Resource 1). There was no overlap of sexual timing (homogamy) within plant for either species. More than one Julian day separated male and female flowering times in 41 out of 50 *J. regia* (82 %) and 9 out of 11 *J. nigra* (81.8 %) trees. As displayed in Fig. 1, during season 2010, the flowering time of black and Persian walnut adult trees ranged from Jd 97 (20 April) to Jd 124 (17 May) and from Jd 117 (10 May) to Jd 156 (17 June), respectively. In 2011, flowering took place from Jd 86 (9 April) to Jd 113 (6 May) for *J. regia* and from Jd 110 (3 May) to Jd 145 (6 June) for *J. nigra*. Thus, the walnut flowering season in 2011 started and ended ten Julian days earlier than in 2010. This shift toward earlier flowering was likely associated with higher average monthly temperature during spring 2011. The mean temperature recorded at the Biagio weather station ranged from 11.7 °C in April 2011, 15.6 °C in May, and 19.72 °C in June, exceeding the corresponding long-term median temperatures calculated over the 18-year period (1992–2010) by +1.7 °C, +1.0 °C, and +1.3 °C. In 2010, the mean blooming duration per individual ranged between 6.27±0.46 (*J. nigra*) and 6.62±0.49 (*J. regia*) days for receptive pistillate flowers and between 4.36±0.67 (*J. nigra*) and 4.34±0.52 (*J. regia*) days for pollen-shedding catkins. As expected, slightly different results were recorded in 2011; pistillate flowers were no longer receptive after 6.00±0.83 (*J. nigra*) and 6.42±0.48 (*J. regia*) days, whereas pollen shedding was completed after 4.20±0.43 (*J. nigra*) and 4.26±0.48 (*J. regia*) days. No significant differences in flowering duration were observed between protandrous and protogynous trees within each species. ANOVA analysis followed by post hoc Tukey's test revealed substantial differences in male

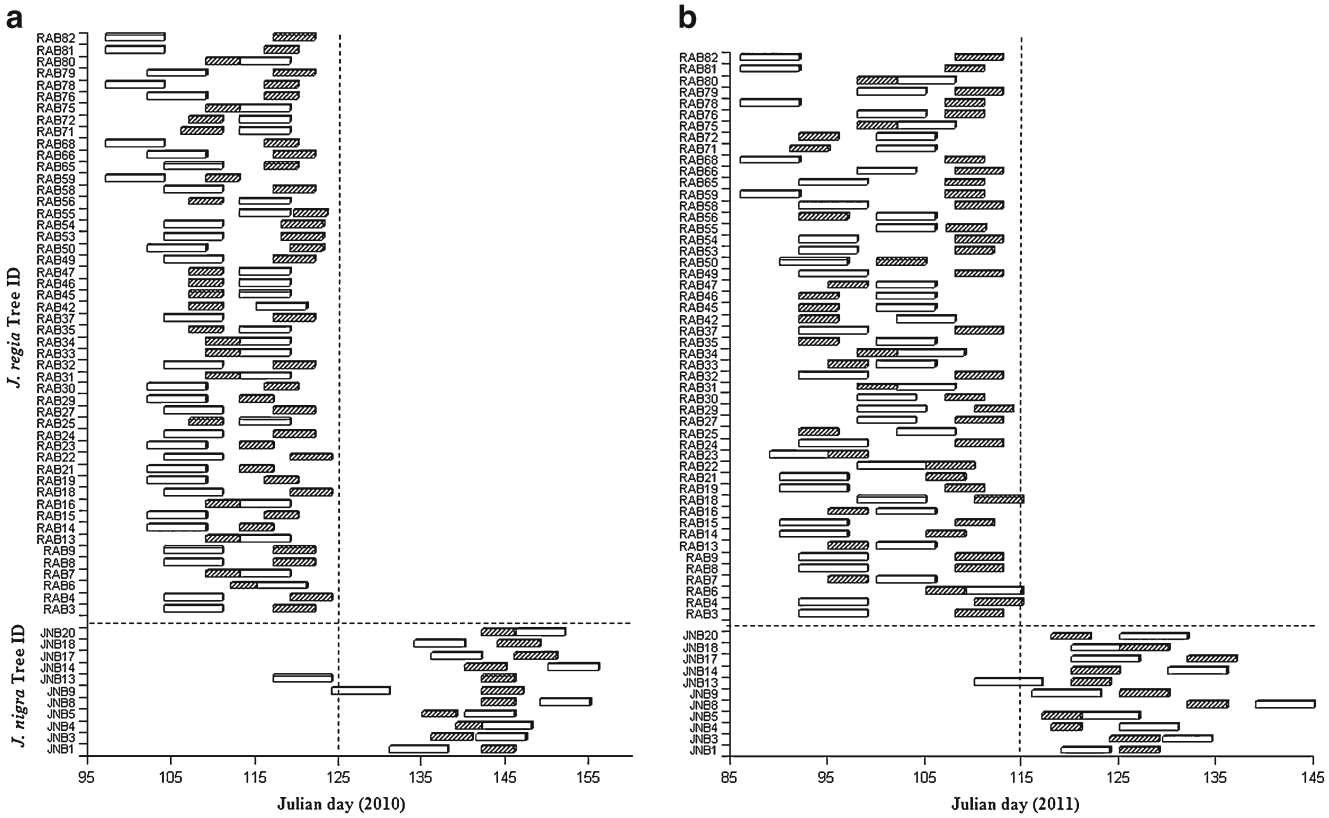

Fig. 1 Flowering period of female (*white bars*) and male (*dashed bars*) inflorescences for 11 *Juglans nigra* (JNB1-JNB20) and 50 *Juglans regia* (RAB3-RAB82) adult trees at the Biagio site in 2010 (**a**) and 2011 (**b**)

($F_{3,\ 56}$=453.61, P<0.0001) and female ($F_{3,\ 56}$=291.01, P< 0.0001) flowering peaks between Persian and black walnut and between the two mating types PG and PA within each species in 2010. Figure 2c–d illustrates the reciprocity and synchronization of the bloom periods of the two mating types within each species during spring 2010. All these results were confirmed during the flowering season in 2011.

Natural hybridization between J. nigra and J. regia

Although Persian walnut bloomed first, about 2–3 weeks prior to black walnut, a small amount of overlap in flowering times between these two species occurred in both 2010 and 2011 (Fig. 1). For instance, in 2010, when a small percentage (4.8 %) of pistillate flowers of PG *J. nigra* began to be receptive, a conspicuous fraction (85 %) of male catkins of PG *J. regia* was still releasing mature pollen (Fig. 2a). By contrast, during the same year, the female flowering of PG- and PA-*J. regia* mating types peaked 29 and 19 days prior to the first day of male flowering of PA-*J. nigra* trees (Fig. 2b). This wide temporal separation prevented the occurrence of reciprocal crosses, i.e., *J. regia*×*J. nigra*. All these results were also observed during the flowering season in 2011.

Therefore, Table 2 presents 2010–2011 synchrony index values calculated for each maternal *J. nigra* tree (female flowering) with respect to the *J. nigra* ($X_{i\text{-}nigra}$) and *J. regia* ($X_{i\text{-}regia}$) groups (male flowering). Within *J. nigra,* the flowering synchrony ranged from 0.000 (JNB9_PG; JNB13_PG) to 0.571 (JNB5_PA) in 2010 and from 0.000 (JNB8_PA) to 0.420 (JNB1_PG) in 2011. Moreover, the degree of synchrony tended to be higher when the female flowering duration of each *J. nigra* tree was compared to male flowering duration of the opposite *J. nigra* mating type (Table 2). High interspecific synchrony was observed only between *J. regia* and the protogynous JNB13_PG *J. nigra* genotype (Table 2). The highest temporal overlap was detected between female flowering of JNB13_PG and male flowering of protogynous Persian walnut with synchrony values ranging from 0.578 ($X_{\text{-}regia\text{-}2010}$) to 0.367 ($X_{\text{-}regia\text{-}2011}$). In particular, receptive stigmas of JNB13_PG overlapped with the pollen shed from 31 and 28 protogynous *J. regia* trees during the first week of the 2010 and 2011 bloom period of *J. nigra,* respectively, with a mean overlap of 4.8 (2010) and 3.3 (2011) days (Fig. 1). Finally, the JNB9_PG *J. nigra* genotype showed unusual low flowering synchrony with both walnut species ($X_{\text{-}nigra\text{-}2010}$=0.00, $X_{\text{-}regia\text{-}2010}$=0.008) in 2010.

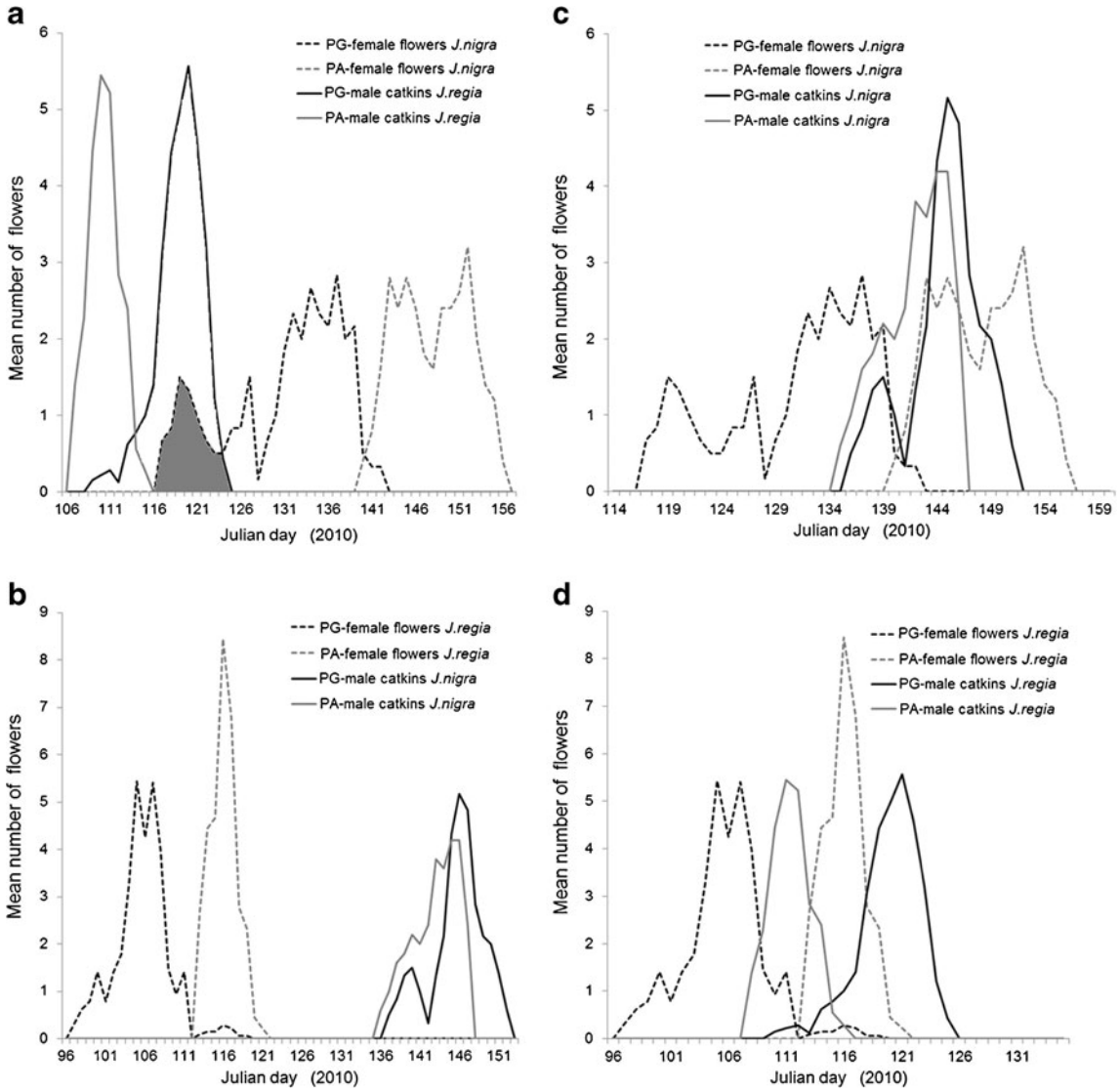

Fig. 2 Mean number of female flowers and male catkins in bloom for each mating type of *J. nigra* and *J. regia* trees in 2010 versus Julian days for four combinations: interspecific cross *J. nigra*×*J. regia* (**a**) and its reciprocal cross *J. regia*×*J. nigra* (**b**), intra-specific cross *J. nigra*×*J. nigra* (**c**), and *J. regia*×*J. regia* (**d**). *PG*, protogynous; *PA*, protandrous. *Filled square*=flowering overlap for interspecific crosses

Variation in floral size of walnut adult trees

Statistically significant differences in flower size were detected between *J. nigra* and *J. regia* (Fig. 3, Online Resource 1). The longest and largest pistillate flowers in the receptive phase were found in the black walnut trees. Mean ovary diameter A (equatorial) and B (polar) was 2.47±0.25 and 2.82±0.53 mm in *J. regia*, by contrast equatorial and polar diameters were 3.27 ±0.26 and 4.39±0.58 mm in *J. nigra* plants, 28.7 % and 55.6 % larger than *J. regia*. The corresponding changes in style and stigma length were great. *J. regia* styles were 1.52±0.24 versus 3.37±0.23 mm for *J. nigra* (+121 %), and *J. regia* stigmas were 5.42±0.40 versus 7.84±0.88 mm for *J. nigra* (+44.6 %). Analogously, mean male catkin length of *J. nigra* (129.60± 14.79 mm) was significantly greater than *J. regia* (75.48± 10.35 mm). The post hoc paired comparison analysis of five floral attributes by Tukey's test confirmed the previous results in a comparison of 11 *J. regia* and 50 *J. nigra* trees (Online Resource 1). Mean ovary size (diameter A, diameter B) and style length did not differ among walnut genotypes within each species. On the contrary, significant variations in stigma length were recorded among black walnut plants, with JNB3_PA (6.50±0.55 mm) and JNB14_PA (6.71±0.57 mm) having shorter stigmas than the remaining germplasm. The mean catkin length varied significantly within each walnut species.

Pollen quality of *J. regia* adult trees

In order to determine if interspecific gene flow between Persian and black walnut was affected by low pollen quality

Table 2 The degree of temporal synchrony of *J. nigra*-female with *J. regia*−(X_{regia}) and *J. nigra*−(X_{nigra}) male flowering calculated for each *J. nigra* maternal tree in spring 2010 and 2011 according to the approach of Augspurger (1983)

Flowering synchrony index	$X_{regia\text{-}2010}$			$X_{nigra\text{-}2010}$			$X_{regia\text{-}2011}$			$X_{nigra\text{-}2011}$		
	Total	PG[a]	PA[a]	Total	PG	PA	Total	PG	PA	Total	PG	PA
Maternal tree ID												
JNB1_PG	0	0	0	0.088	0	0.145	0	0	0	0.420	0.250	0.533
JNB3_PA	0	0	0	0.500	0.542	0.023	0	0	0	0.183	0.266	0.472
JNB4_PA	0	0	0	0.543	0.685	0.400	0	0	0	0.329	0.485	0.171
JNB5_PA	0	0	0	0.571	0.542	0.600	0	0	0	0.357	0.371	0.343
JNB8_PA	0	0	0	0.057	0.114	0	0	0	0	0	0	0
JNB9_PG	0.008	0.012	0	0	0	0	0	0	0	0.314	0.142	0.429
JNB13_PG	0.370	0.578	0	0	0	0	0.235	0.367	0	0.014	0	0.024
JNB14_PA	0	0	0	0.029	0.057	0	0	0	0	0.171	0.200	0.143
JNB17_PG	0	0	0	0.314	0.107	0.452	0	0	0	0.413	0.437	0.396
JNB18_PG	0	0	0	0.186	0	0.309	0	0	0	0.367	0.291	0.417
JNB20_PA	0	0	0	0.214	0.400	0.028	0	0	0	0.300	0.450	0.150

[a] Flowering synchrony index computed in relation to protogynous (PG) and protandrous (PA) mating types of walnut trees

of putative donors, the correct development of male gametophytes, pollen viability, and germination were evaluated in a subset of six *J. regia* protogynous trees showing male flowering overlap with female flowers of JNB13_PG, a protogynous *J. nigra* genotype. Male sporogenesis and gametogenesis in all Persian walnut plants proceeded normally through stage 1 (compressed tetrads, Fig. 4a), 2 (tetrads, Fig. 4b), and 3 (uninucleate pollen grains, Fig. 4c). Normal completion of meiosis was observed in *J. regia* anthers spanning the stages corresponding to 10–13-mm-long catkins (Fig. 4a, b). Morphologically irregular tetrads were not formed and the subsequent normal early pollen development was detected in catkins about 20-mm long (Fig. 4c). No abnormal male gametes were observed, although we checked for irregularly shaped pollen grains and the separation of cytoplasm from the cell wall. Accordingly, the number of viable and semi-viable pollen grains (stained) was high for all six plants, ranging from 81.7 % (RAB82_PG) to 91.0 % (RAB9_PG), with a mean percentage of 86.6±3.7. Pollen germination was also checked to confirm previous results. All samples showed greater than 50 % pollen germination in vitro, varying from a minimum of 51.86 % (RAB27_PG) to a maximum of 64.14 % (RAB37_PG) with mean percentage of 57.65±4.72. With respect to pollen viability and germination, all six putative Persian walnut paternal trees could be considered ideal pollen donors.

Seed arrays from *J. nigra* adult trees

Seeds (n=1148) produced by open pollination of the 11 *J. nigra* adult trees were collected during autumn 2010 and extensively evaluated (Online Resource 2). Among *J. nigra* plants, there was statistically significant variation in all seed traits analyzed in this study. The number of seeds produced per tree varied from 15 for JNB9_PG, to 201 for JNB14_PA genotype. Tukey's test also revealed differences in seed

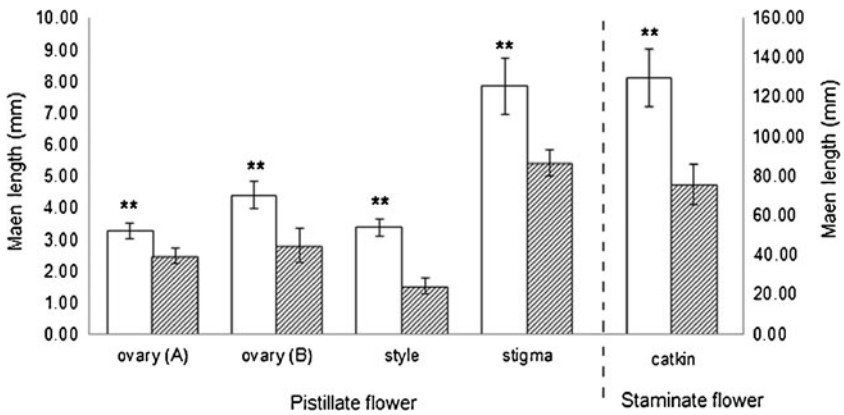

Fig. 3 Morphological characters of receptive pistillate flowers and mature catkins collected from 11 *Juglans nigra* (*empty square*) and 50 *Juglans regia* (*filled square*) adult trees at Biagio during spring 2010: ovary size (mean length of equatorial diameter A and polar diameter B±SD), mean length of style (±SD), stigma (±SD), and catkin (±SD). Statistical significance was tested by Student's *t* test with $^{**}p<0.0001$

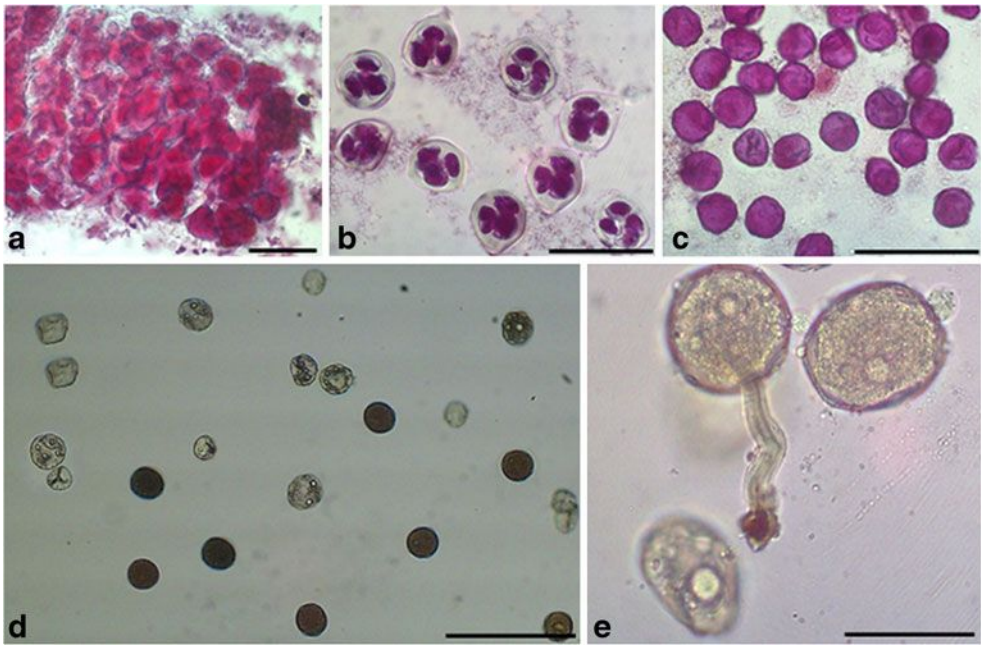

Fig. 4 Microsporegenesis and early pollen development in *J. regia* species. **a** Compressed tetrads of microspores in a 10-mm-long catkin bud (*stage 1*). **b** Tetrads release from 13 mm-long catkin (*stage 2*). **c** Uninucleate pollen grains in a 20 mm-long catkin (*stage 3*). **d** mature pollen from 75 mm-long catkin (*stage 4*) showing different viability: purple pollen grain=viable, light red-pink pollen grain=semi-viable, non-dyed pollen grain=non-viable. **e** Pollen grain germination and tube growth in vitro. *Bars* 50 μm in all panels

weight among black walnut seed cohorts (Online Resource 2). As expected, seed mass was positively related to three UPOV morphological parameters, seed thickness (r=0.91, P<0.01), seed width (r=0.91, P<0.01), and seed length (r=0.68, P< 0.05). The percentage of germinated seeds also varied from 13.40 % (8 seedlings) for JNB17_PG to 72.40 % (34 seedlings) for JNB18_PG progeny array, with a mean of 36.75 % on a total of 422 walnut seedlings germinated under glasshouse conditions (Table 3). Seed germination was negatively correlated to seed number per tree (r=−0.60, P<0.05) and positively correlated to seed size assessed as seed weight (r= 0.67, P<0.05), seed thickness (r=0.7048, P<0.05), and seed width (r=0.6537, P<0.05). Thus, we observed that when seed production decreased, the seed mass/size and percentage of seed germination significantly increased. Finally, there were no significant relationships between any seed trait and the inter- and intra-flowering synchrony computed for each *J. nigra* plant during 2010. However, although there wasn't a

Table 3 Fingerprinting analysis of 422 seedling offspring of 11 open-pollinated *J. nigra* progenies and subsequent hybrid identification by STRUCTURE (Pritchard et al., 2000) and NewHybrids (Anderson and Thompson, 2002) Bayesian-assignment tests using nine SSR markers (paternity analysis of hybrid offspring based on most-likelihood approach (Marshall et al. 1998; CERVUS software))

Maternal tree ID	Total number of offspring	Bayesian assignment			Paternity analysis of hybrid offspring	
		J. regia	*J. nigra*	F1 hybrid	Not-excluded fathers (cases)	Most-likely fathers
JNB1_PG	58	–	58	–		
JNB3_PA	56	–	56	–		
JNB4_PA	52	–	52	–		
JNB5_PA	38	–	38	–		
JNB8_PA	69	–	69	–		
JNB9_PG	8	–	8	–		
JNB13_PG	15	–	12	3	RAB8_PG (2), RAB24_PG, RAB27_PG, RAB31_PA, RAB32_PG, RAB53_PG (2), RAB54_PG (2), RAB55_PG, RABB58_PG, RAB66_PG, RAB72_PA, RAB81_PG, RAB82_PG (3)	RAB27_PG RAB54_PG RAB55_PG
JNB14_PA	52	–	52	–		
JNB17_PG	8	–	8	–		
JNB18_PG	34	–	34	–		
JNB20_PA	32	–	32	–		
Total	422	–	419	3		

significant correlation between seed number and flowering synchrony, a clear tendency was observed: JNB9_PG and JNB13_PG genotypes with the lowest seed production (Online Resource 2) exhibited no flowering overlap with *J. nigra* and positive interspecific flowering synchrony with protogynous *J. regia* trees, i.e., high ($X_{\text{-regia-2010}}$=0.578) for JNB13_PG and low ($X_{\text{-regia-2010}}$=0.012) for JNB9_PG.

Detection of first-generation hybrids among progeny arrays

The nine selected microsatellites amplified in all 61 adult walnut trees and 422 seedling offspring at the Biagio site. A total of 77 alleles were amplified with an average of 8.6 alleles per locus, ranging from 6 in WGA1, WGA9, and WGA331 to 13 in WGA276 (Table 1). The final classification of the adult trees and the identification of *J.* × *intermedia* genotypes among 422 seedlings (11 progenies) was conducted by comparing their SSR profiles against 384 *J. nigra*, 80 *J. regia,* and 205 F1 *J.* × *intermedia* genotypes (reference set) that had already been characterized by Pollegioni et al. (2009, 2011) in Italy. The Principal Coordinate Analysis (PcoordA) performed on the Band–Sharing similarity coefficient computed using nine SSR loci provided preliminary insights into the relationships between 1,152 total individuals. Distinct *J. nigra* and *J. regia* clusters and the presence of several intermediate individuals were observed (Fig. 5). The first and second axis, which accounted for 19.01 % and 7.93 % of the variance, respectively, clearly separated the *J. regia* cluster composed of 80*J. regia* reference samples and 50*J. regia* RAB trees from the *J. nigra* cluster that included 384*J. nigra* reference samples, 11*J. nigra* JNB, and 419 progeny seedlings. The remaining three offspring genotypes and 205 F1 *J.* × *intermedia* reference samples were incorporated in third group located at intermediate position between black and Persian walnut.

The Bayesian admixture analysis of the reference sets performed with STRUCTURE demonstrated maximum accuracy when assigning individuals to each of the three classes: *J. nigra*, *J. regia,* and hybrids. In particular, using K=2 as most likely number of genetic clusters and a threshold of Q=0.90, we distinguished 203 F1 walnut hybrids ($0428 \le Q_1 \le 0.534$) from 389*J. nigra* ($Q_2 \ge 0.976$) and 80*J. regia* ($Q_1 \ge 0.950$) purebreds. In addition, 50*J. regia* (RAB) and 11*J. nigra* (JNB) individuals from Biagio exhibited more than 0.99 probability of belonging to the species expected. Of the 422 seedlings from 11*J. nigra* progeny arrays (Table 3), the majority 419 (99.28 %) showed a high affinity to the *J. nigra* cluster ($Q_2 \ge 0.90$) and only three seedlings displayed hybrid ancestry ($0475 \le Q_1 \le 0.516$). The Bayesian-assignment analysis by NewHybrids software confirmed previous results obtained from PcoordA and STRUCTURE. All purebred genotypes were assigned with high posterior probability ($q_i \ge 0.99$) to the same purebred category as STRUCTURE. Using a threshold of q_i>0.90 (strict level), 100 % of F1 hybrids in the reference set were assigned to the correct genotype frequency class. NewHybrids software also classified 419 progeny seedlings as *J. nigra* and three seedlings as first-generation walnut hybrids with $q_i \ge 0.99$. Analysis of the progenies revealed the presence of one hybridogenic *J. nigra* mother tree (Table 3). The three F1 hybrid offspring identified in our walnut collection were grown from seeds harvested from *J. nigra* JNB13_PG. Thus, the proportion of seedlings resulting from crosses with *J. regia* was 20 % (3/15) for the family JNB13_PG and 0 % for the remaining ten *J. nigra* progenies. Finally, the proportion of F1 hybrids in progeny arrays had a positive relationship (r=0.999, p<0.01) with the degree of synchrony between female flowering of each *J. nigra* tree and male flowering of PG−*J. regia* calculated during 2010. No significant correlation was found between any seed trait and hybrid rate.

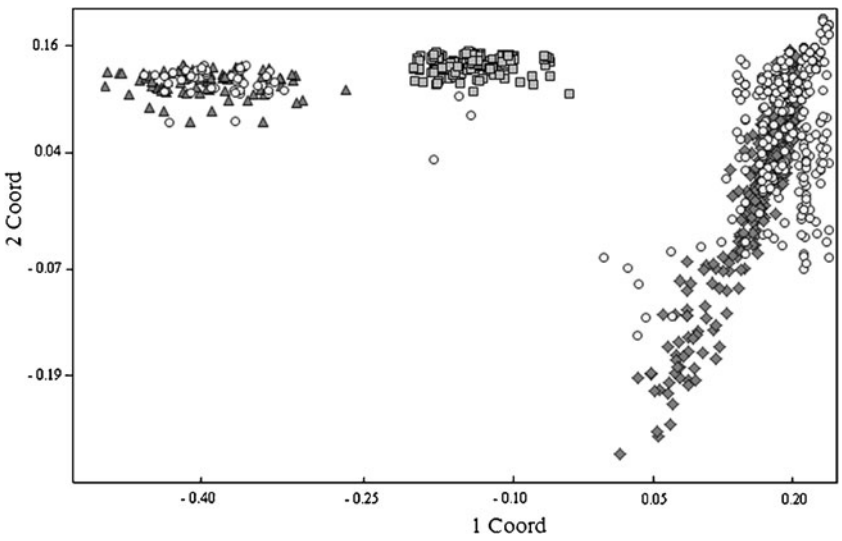

Fig. 5 Principal coordinate analysis of 1,152 *Juglans* individuals based on genotypic similarity as determined by Band–Sharing coefficients based on nine SSR loci. *Filled triangle J. regia* (N=80), *filled diamond J. nigra* (N=384), *filled square* diploid hybrids (N=205) used as a reference set and *empty circle* 482 walnut genotypes from the Biagio site (adult walnut plants N=61; *J. nigra* offspring N=422)

Paternity analysis of hybrid seedlings

The genetic diversity parameters for each locus were computed separately for *J. regia* (50 RAB) and *J. nigra* (11 JNB, 419 seedlings) (Online Resource 3). The microsatellite loci revealed a high level of variability in the tested samples. No significant deviation from Hardy–Weinberg equilibrium (HWE) was found in *J. nigra* genotypes, and in the *J. regia* group, only WGA321 showed significant deviation from HWE. Across all nine loci, PI_{unb} and PI_{sib} values ranged from 1.6×10^{-6} (*J. nigra*) to 5.9×10^{-6} (*J. regia*) and from 2.6×10^{-3} (*J. nigra*) to 4×10^{-3} (*J. regia*), respectively, indicating that the probability that two unrelated individuals would share the same genotypes or the probability that two full-sibs will have identical genotypes were extremely low in most cases. 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 groups. The combined power of exclusion ranged from 0.866 (*J. nigra*) to 0.818 (*J. regia*) for EP1 and from 0.977 (*J. nigra*) to 0.962 (*J. regia*) for EP2, indicating that the levels of polymorphism were sufficient for the subsequent parentage analysis of hybrid seedlings beyond any reasonable doubt.

Results of paternity assignment indicated that the three F1 hybrid seedlings resulted from crosses of JNB13_PG with *J. regia* trees growing inside the Biagio plantation (Table 3). According to the simple exclusion method based on the Mendelian rules of inheritance, eleven protogynous and two protandrous *J. regia* plants were the not-excluded fathers. The application of the approach of Marshall et al. (1998) permitted the identification of three protogynous Persian walnuts, RAB27_PG, RAB54_PG and RAB55_PG, as the most likely pollen donors with 95 % critical values of Δ (Table 3). The mean planting distance of pollen donors from female partner was 12.36±6.55 m, ranging from 6 m (RAB55_PG) to 19.1 m (RAB27_PG). The frequency of pollen dispersal events inferred from direct paternity showed a general decline with distance between the mother and the assigned fathers, but the small number of hybrids detected prevented further analysis of this pattern.

Discussion

The phenology data recorded during the two-year observations of 11 *J. nigra* and 50 *J. regia* adult trees revealed the complete temporal separation (at least one day) of female and male functions within plant. Our walnut trees exhibited a phenotypic dimorphism of PA and PG mating types (morphs) typical of *Juglans* spp., including *J. cinerea* (Stout 1928) *J. ailantifolia* (Kimura et al. 2003), *J. mandshurica* (Bai et al. 2007). Fluctuations in the weather conditions may influence the rate of walnut flower development. In general, staminate inflorescences emerged and catkins shed pollen earlier with rising temperatures (Funk 1970). Hot weather during spring 2011 promoted earlier blossom at the Biagio site (−10 Jd), slightly reduced the mean male and female flowering duration of *J. regia* and *J. nigra*, but did not affected morph classification of trees. As discussed by Bertin and Newman (1993), dichogamy represents an evolutionary mechanism to encourage outcrossing and reduce or prevent self-pollination. Notably, genetic models predicted a stable polymorphism with both mating types at equal frequency for heterodichogamous species. In particular, 1:1 balance of morph frequencies in a population should be achieved when the mating system promotes inter-morph pollen transfer (disassortative mating) with nearly 100 % outcrossing and when the two morphs present equal fitness (Bai et al. 2007). In this study, the sexual functions of the two morphs were synchronous and reciprocal within each walnut species. Similarly, protogynous and protandrous *J. nigra* individuals co-occurred at the Biagio plantation at the expected 1:1 ratio. On the contrary, the ratio of morphs of *J. regia* individuals was biased to protogyny. Although we can't rule out the idea that the statistical deviation from the expected ratio might be related to genetic control of the heterodichogamy (Kimura et al. 2003), we postulated that human selection has influenced morph ratio in this artificial *J. regia* population.

Field observation of flowering times indicated that morph ratio coupled with flowering synchrony may play a role in gene flow between Persian and black walnut. In our mixed stand, we found considerable temporal displacement in flowering times between the two species in 2010 and 2011, in agreement with annotations of Funk (1970). These data could partly explain why natural hybridization in walnut is considered so uncommon. Divergent flowering peaks were a strong isolating barrier in both animal-pollinated (Marques et al. 2007) and wind-pollinated plants (Ortiz-Barrientos and Rieseberg 2006) preventing pollen-mediated gene flow from occurring. Flowering asynchrony has been identified as essential mechanism for maintaining overall “genetic identity” and preventing interspecific cross in *Populus* (Vanden Broeck et al. 2005), *Eucalyptus* (Barbour et al. 2006), and *Quercus* spp.(Craft et al. 2002). Bridging the initial gap between two divergent species through the formation of F1 hybrids was considered the more difficult step. In the present study, the proportion of pistillate receptive flowers that could receive hetero-specific pollen was quite small, being restricted to the short interval when outliers of each species flowered simultaneously. Interspecific overlap occurred, but it was only between the end-tail of staminate flowering in protogynous *J. regia* and the beginning of pistillate flowering of protogynous *J. nigra* trees. We also postulated that the period of

interspecific overlap was further extended by the fact that *J. regia* was strongly protogynous at the Biagio site. Therefore, the preferential direction of hybridization was a pollination of few early-flowering PG *J. nigra* by late flowering PG *J. regia* trees. Based on our blooming data, natural hybridization in the opposite direction (*J. regia*×*J. nigra*) seems impractical because none of the *J. regia* stigmas were receptive when staminate flowers of *J. nigra* began shedding mature pollen. Asymmetries in reproductive isolation among plants appeared to be common and taxonomically widespread. In many cases, hybridization is asymmetrical, and one species is more often the maternal parent of hybrid offspring (Tiffin et al. 2001).

Assessment of the synchrony of flowering in *J. nigra* and *J. regia* provided a detailed picture regarding the possibility of interspecific cross-pollination. Within 2010–2011, we could distinguish 28 (56 %) protogynous *J. regia* whose catkins always released mature pollen when one (0.09 %) protogynous *J. nigra* tree, JNB13_PG, exhibited receptive stigmas; thus they were considered good candidates as paternal and maternal hybridogenic plants, respectively. In particular, the black walnut JNB13_PG displayed an early-flowering habit with receptive stigmas present the first 8 days of the 6 weeks (2010) or 5 weeks (2011) of *J. nigra* bloom period.

In this study, a significant positive correlation between hybridization rate and individual interspecific flowering synchrony was also detected. Fingerprinting analysis of eleven *J. nigra* progeny arrays by SSR markers permitted the identification of three first-generation *J.*× *intermedia* among 422 offspring and confirmed the identity of the hybridogenic mother tree JNB13_PG. The three F1 hybrids were sired by three protogynous Persian walnuts (RAB27_PG, RAB54_PG, RAB55_PG) selected as possible pollen donors for interspecific crosses. Nevertheless, taking into consideration the ample flowering synchrony between JNB13_PG and local protogynous *J. regia* plants, the proportion of hybrid progeny (3/15) and the number of seed (25) produced by open pollination of JNB13_PG during season 2010 was surprising low. Our findings proved that the degree of flowering overlap may be a rough indicator of hybridogenic ability in walnut. The temporal overlap between pollen emission from *J. regia* and the stigma receptivity in *J. nigra* is a necessary factor but not sufficient to explain the actual incidence of hybrids.

Pre- and post-zygotic barriers related to pollen quality and quantity, pollen tube growth, zygote formation and to embryo development tend to reduce seed yield and hybrid production (Rieseberg and Carney 1998). In this study, two black walnut trees, JNB13_PG and JNB9_PG, with the lowest seed production exhibited no flowering overlap with other *J. nigra* but positive interspecific flowering synchrony, high for JNB13_PG and very low for JNB9_PG. Both black walnuts flowered profusely during 2010 but a high incidence of pistillate flowers abscission probably caused by lack of pollination was noted (data not shown). Separation of flowers at the zone between the ovary and peduncle occurred at a late stage of development, about 3 weeks after bloom. This feature is typical of non-fertilized pistillate flowers (Catlin et al. 1987). In addition, 15 (100 %) and 22 (88 %) seedlings grown from seeds harvested from JNB9_PG and JNB13_PG, respectively, were classified as *J. nigra* and sired by black walnut trees located outside the study site (data not shown). Clearly, our research site was not spatially isolated from other *Juglans* plantation, and walnut pollen regularly moves for long distances. Even if *J. nigra* plants are not common near the Biagio site, immigrant black walnut pollen acted as an effective competitor during fertilization at a time when local *J. regia* pollen was available. Therefore, we posit that at the Biagio site, JNB9_PG may suffer from insufficient pollination, whereas JNB13_PG received large pollen loads (mainly from *J. regia*) but exhibited a conspecific pollen preference. The correct development of male gametophytes and high pollen viability and germination observed in six *J. regia* plants potentially involved in the interspecific crosses ruled out the possibility that low pollen quality contributed to depressed seed and hybrid production.

Differences in floral size were found between the two walnut species. The total length of pistillate flowers (from stigma to base of ovary) in receptive *J. nigra* was ~15.6 mm, about 5 mm longer than *J. regia*. In addition, pollen grains of Persian and black walnut were roughly spherical in shape but they differ in diameter, ranging from 33 to 44 μm (Mert 2010) and from 31 to 68 μm (Kapp et al. 2000), respectively. Differences in flower size, in particular style length disparities, have been proposed as the cause of some previous failed interspecific hybridization attempts in forest trees such as *Eucalyptus* (Potts and Dungey 2004) and *Populus* spp. (Vanden Broeck et al. 2005). Observation of floral morphology indicated that a higher degree of hybridization success was realized between species with similar flower size. A positive genetic association (gametic-phase disequilibrium) among genes regulating flora organ size was observed in plants (Sarkissian and Harder 2001). The potential length of the pollen tube was directly related to the length of the style. Large pollen had a higher probability of siring seeds in pistils with long styles because of its faster germination and faster or more prolonged pollen-tube growth. Hence, disparities of flower size might prevent pollen tubes of small-flowered *J. regia* from reaching and fertilizing the ovule of longer-flowered *J. nigra*. This unilateral structural barrier may be an impediment for producing *J.*× *intermedia* at high frequency. Furthermore, we can't rule out the possibility that the low frequency of fertilization between Persian and black walnut may also be caused by inefficient pollen–pistil recognition during germination and

pollen tube elongation (pre-fertilization incompatibility). Successful fertilization depends on specific pollen–pistil interactions, and only "compatible" pollen grains are able to complete the passage through stigma, style, and ovary (Tiffin et al. 2001). Pollen tube growth takes place in the extracellular matrix of the stigmatic and stylar transmitting tissues and along the ovule surface. According to Calzoni et al. (1990), the pattern of soluble cytoplasm, membrane, and cell-wall proteins of *J. nigra* and *J. regia* pollen vary quantitatively and qualitatively. These differences likely reflected differences in enzymatic activities critical for hydration during pollen germination, for adhesion and penetration through the stigmatic and stylar transmitting tissue, and for proper pollen tube guidance. The presence of *J. regia* pollen tube abnormalities and pollen tube arrest in the *J. nigra* pistil is currently under evaluation.

In conclusion, our findings indicated that natural hybridization between Persian and black walnut occurred at low frequency even when conditions to encourage hybridization were highly favorable. As expected, these two species tended to remain reproductively isolated. This study revealed for the first time that the substantial disjunction in flowering time observed in the field may affect interspecific gene flow between *J. regia* and *J. nigra* species, but also, additional prezygotic barriers such as differences in floral size and conspecific pollen advance may also be present.

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