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REVIEW

Walnut: past and future of genetic improvement

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Abstract

Persian or English walnut (*Juglans regia* L.), the walnut species cultivated for nut production, is one of the oldest food sources known. Persian walnuts, native to the mountain valleys of Central Asia, are grown worldwide in temperate areas. World production exceeds three million tons since 2012, mostly provided by China, the USA, and Iran. Despite very ancient culture of walnut species (*Juglans* spp.), breeding actually started in the twentieth century. Using a range of methodologies, from morphological markers to the most recent advances in genome analysis, many genetic studies of walnut have been conducted during the past 30 years, including examination of diversity, determination of relationships within or among germplasm collections and populations, phylogenetic and origin elucidation, genetic map construction, and biotic or abiotic stress investigations. The genetic improvement of walnut has undergone great evolution. The producing countries of the Middle East have widely studied morphological characteristics of walnut. The USA and France, for example, are behind important cultivar releases such as "Chandler" and "Franquette." Finally, genomics represents a major breakthrough in walnut improvement, in particular by recent sequencing of both chloroplast and nuclear genomes. This review summarizes worldwide molecular and "omics" studies and gives an overview of the main walnut breeding programs.

Keywords Genetic improvement · Breeding program · Molecular markers · Genomics · Walnut · *Juglans* spp.

Introduction

Nuts

Tree nuts are one of the oldest sources of food for man, birds, and wild animals (Woodroof 1967), and their history is very rich. Ancient fossils prove their presence on earth long before man arrived (Jaynes 1969). References to nut trees in ancient literature are numerous. Theophrastus, Greek philosopher born 370 B.C., referred to walnut and mentioned hazelnuts as growing in the mountains of Macedonia (Jaynes 1969). Furthermore, branches and flowers have been used on religious occasions since antiquity and several tree nuts, like almonds and pistachios, were mentioned in the Bible, Genesis 43:11 and Song of Solomon 6:11. It is mentioned that Persian walnuts (*Juglans regia* L.) were cultivated in King Solomon's garden, and almonds were depicted for ornamentation of the candlesticks in the Temple (Moldenke and Moldenke 1952). Nowadays, "nuts" are still a valuable food resource, widespread in the northern hemisphere due to their very high food and ornamental value (Jaynes 1969).

In botany, the word "nut" is very ambiguous. According to the *American Heritage Dictionary of the English Language, 5th Edition, 2011*, "a nut is an indehiscent fruit having a single seed enclosed in a hard shell, such as an acorn or hazelnut," or "any of various other usually edible seeds enclosed in a hard covering such as a seed coat or the stone of a drupe, as in a pine nut, peanut, almond or walnut." Finally, it seems that a consensus has been accepted by the scientific community in which a nut is a simple dry fruit with one seed in which the ovary wall becomes increasingly hard as it matures, and where the seed remains unattached within the ovary wall (Woodroof 1967; Jaynes 1969). All nuts are indehiscent and do not open at maturity. True nuts are produced for example by some species of the order *Fagales* (beech, chestnut, oak, hazelnut, etc.). However, in a general context, a wide variety of dried seeds are also called "nuts" by extension because of their shell (Jacquenet and Moneret-Vautrin 2007)—this is actually the

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culinary definition. A botanical classification of species producing nuts was proposed by Jacquenet and Moneret-Vautrin (2007) (Fig. 1).

With the development of molecular biology in the 1990s, numerous works were undertaken in order to improve the characteristics of nuts, particularly of walnuts. These studies facilitated accumulation of knowledge regarding biochemistry of walnuts, their genetic diversity, and the genetic determinants of agronomic traits of interest such as yield or disease resistance. There are still efforts in progress, particularly thanks to the team at the University of California-Davis that sequenced the Persian walnut genome in April 2016 (Martínez-García et al. 2016).

Taxonomy and origin

Walnut is defined at the nut of any tree of the genus *Juglans* spp., part of the subclass *Rosidae* (Germain et al. 1999). Walnuts are among the most produced "nuts" in the world alongside almonds, hazelnuts, pistachios, and cashew nuts, according to the International Nut and Dried Fruit Council in 2015. The Persian walnut tree (*Juglans regia* L. or English walnut) is a wide spread monoecious tree species of the order *Fagales* (formerly, in the order *Juglandales*) and the family *Juglandaceae*. This family includes about 50 species of 11 genera of which *Carya* (hickory tree), *Pterocarya* (wingnut tree), and *Juglans* are the major members (Rehder 1947). The genus *Juglans* includes more than 20 species divided into four sections: *Trachycaryon* (butternut, a single species, *J. cinerea*), *Cardiocaryon* (heartnuts), *Rhysocaryon* (black walnuts, notably *J. nigra*), and *Dioscaryon* containing only *J. regia* (Manning 1978). This classification was confirmed with a study of the nucleotide sequences of the chloroplast gene *matK* and the internal transcribed spacers (ITS) and 5.8S gene of the nuclear ribosomal DNA (Stanford et al. 2000) (Table 1). *J. regia* is widely disseminated and grown in many temperate regions of Europe, North and South America, South Africa, Asia, Australia, and New Zealand (Aradhya et al. 2006). All species of the genus *Juglans* are diploid with $2n = 2x = 32$ chromosomes (Woodworth 1930).

Fossil evidence (Manchester 1987) traces the origin of genus *Juglans* to the Middle Eocene in North America (from ~48 to ~38 million years ago), well after the now extinct first tree-like plant, *Archaeopteris*, which likely first appeared during late Devonian (~382 mya). Subsequent expansion and diversification of *Juglans* from North America was made possible by the Bering land bridge during Middle Tertiary (from ~66 to ~3 mya) and the North Atlantic land bridge during late Eocene (~38 to ~33 mya). In addition, there was an adaptation to Neogene cool climate (~23 to ~3 mya) (Wolfe 1978) and other expansions towards Southeastern Europe and Central and South America during late Myocene (~12 to ~5 mya). From studying chloroplast DNA (cpDNA) intergenic spacer sequences, it seems that the division within genus *Juglans* (*Rhysocaryon*, *Cardiocaryon*, and *Dioscaryon* sections only) occurred during a period ranging from ~50 to ~40 mya, which matches with fossil analyses (Aradhya et al. 2006, 2007) (Fig. 2). The cradle of Persian walnut domestication is thought to be located in Central Asia (Zeven and Zhukovsky 1975), particularly the foothills of the Western Himalayas from the Kashmir region to Tajikistan and Kyrgyzstan. It first spread to the west, namely Uzbekistan, north Iran, Caucasus region, and

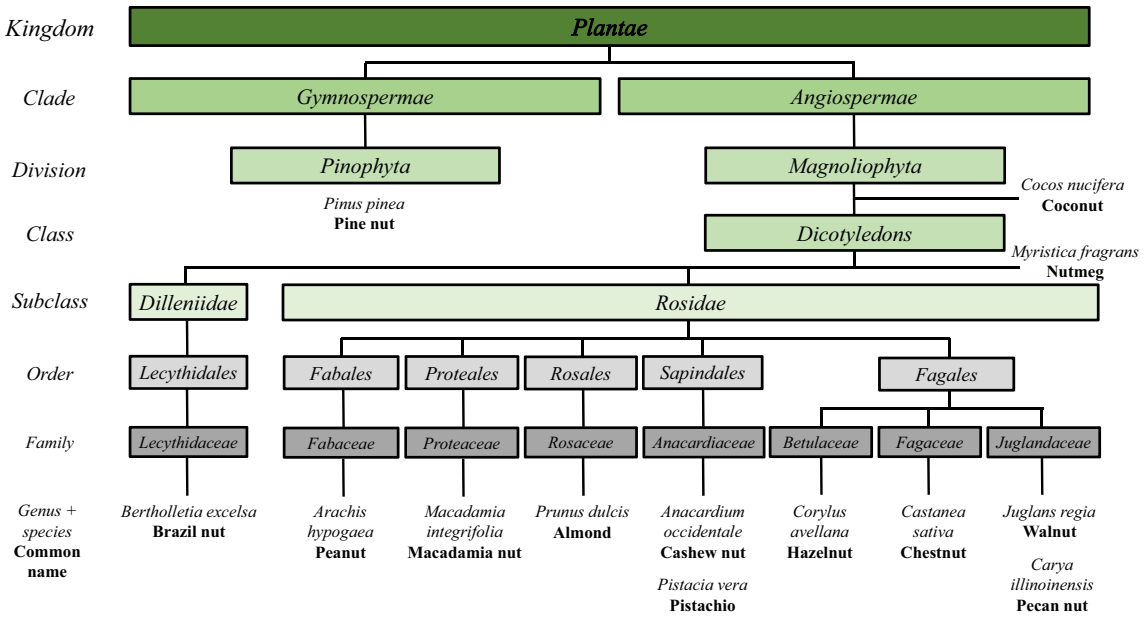

Fig. 1 Botanical classification of nuts adapted from Jacquenet and Moneret-Vautrin (2007) and according to the Angiosperm Phylogeny Group (APG III 2009)

Table 1 Short description of species of the genus *Juglans* L. adapted from Germain et al. 1999 and other sources

Section		Botanical characteristics	Species	Vernacular name	Main information
Common walnut	Dioscaryon	Leaves with small number of few or not dentate and hairless leaflets (7/9), absence of lacunae in the nut shell with thin primary and secondary walls, dehiscent husk when mature	*Juglans regia* L.	Persian or English walnut	Cultivated for nut and wood in the northern hemisphere (Europe, Asia, North America) and the southern hemisphere (Australia, New Zealand, South Africa, South America)
			Juglans sigillata Dode	Iron walnut	Not easily distinguished from *J. regia*, native to China, few information but nut is oval-shaped and tree used for its wood, commonly found in Yunnan province in China
Black walnut	Rhysocaryon	About 16 species from the Americas, leaves with dentate leaflets (9/25), nut with thick shell and presence of lacunae with well-developed and lignified primary and secondary walls, not dehiscent hairless husk or slightly pubescent	*Juglans nigra* L.	Eastern black walnut	Spontaneous in Eastern USA, introduced in Europe at the beginning of the XVIIth century for ornamental use and then for rootstock, bark and wood darker than *J. regia*, 25 to 40-m high tree, 13/23 leaflets lanceolate/narrow/elongated/finely dentate at their sides, nuts regularly larger than longer and slightly flattened, presence of black lacunae in the nut shell and difficult to break, pronounced taste appreciated in Northern America for ice creams and cakes, more resistant to extreme cold than *J. regia* but more susceptible to spring frosts (early budding), tolerant to *Armillaria mellea* rot, to ink disease *Phytophthora cinnamomi* and relatively not susceptible to crown gall *Agrobacterium tumefaciens.* Now used for wood and nut is correct
			Juglans hindsii Jeps.	Northern California black walnut or Hinds' black walnut	Native to Northern California, few in France, 20 to 30-m high tree, 15/21 leaflets lanceolate/narrow/finely dentate, green-blue, almost smooth nut, early budding, late flowering, appreciated for its wood, widely used in California as rootstock ("Paradox" rootstock is a hybrid from *J. hindsii* pollinated by an English walnut), tolerant to rot but susceptible to crown gall, also for ornamental use with small edible nut
			Juglans microcarpa Berl.	Texas or little black walnut	Native to Texas and Northern Mexico, semi-bushy, 10 m high at most, globulous nuts with thick shell and presence of lacunae, very furrowed, distinguished from other black walnuts by its small size (1.5 cm diameter), 15/23 leaflets dentate/small/narrow/lanceolate, lower side oftenly fluffy, can interbreed with *J. major* and *J. nigra*
			Juglans californica S. Wats.	Southern California black walnut	

Table 1 (continued)

Section	Botanical characteristics	Species	Vernacular name	Main information
				Native to Southern California, 11/15 leaflets small/lanceolate/narrow like *J. microcarpia* but hairless on lower side even juvenile, small globulous nuts, no more than 2.5 cm diameter, susceptible to roots diseases so not used as rootstock, ornamental use
		Juglans major Heller	Arizona black walnut	Native to Southwestern USA and Northern Mexico, up to 20 m high, stout trunk, distinguished from *J. microcarpa* by bigger nuts and larger leaflets fewer in number (9/15) more coarsely dentate, nuts with 2/3 cm diameter, less furrowed than *J. nigra*, less cracked bark, leaves slightly green-blue, used as rootstock as *J. nigra*, edible nuts and used as carpentry wood and firewood
		Juglans australis Griseb. (*J. brasiliensis* Dode)	Argentine or Brazilian walnut	Native to forests of Argentina and Bolivia, up to 20 m high, more resistant to frost than *J. regia*, edible nuts
		Juglans boliviana (C. DC.) Dode	Bolivian or Peruvian walnut	Native to Northern Bolivia and Central and Southern Peru, few information
		Juglans friburgensis		Native to Breisgau, area in Southwest Germany; few information
		Juglans hirsuta Mann.	Nuevo León walnut	Native to Mexico, 14/23 lanceolate leaflets, few information
		Juglans jamaicensis C. DC. (*J. insularis* Griseb.)	West Indies walnut	Found in Cuba, Dominican Republic, Haiti, and Puerto Rico but not native to Jamaica; can reach 25 m high; edible nuts of 2/3 cm diameter and wood used like *J. nigra*
		Juglans mollis Engelm.	Mexican walnut	Native to Mexico, up to 15 m high, edible nuts, few information
		Juglans neotropica Diels	Andean, Ecuadorian or Colombian walnut	Found in Colombia, Ecuador, and Peru; can reach 40 m high; reddish-brown bark; oval-shaped canopy; large leaves (40 cm); nuts eaten in local Ecuadorian market; more difficult to crack than *J. regia*; one of the most expensive wood because of its durability (floor and decoration)
		Juglans olanchana Standl. & L.O. Williams	Cedro Negro	Found in Costa Rica, Guatemala, El Salvador, Honduras, Mexico, and Nicaragua; can reach 40 m high; darker leaves on upper side; husk used to color leather; brown wood easy to work used for fine furniture and lute-making; edible nuts
		Juglans soratensis Mann.		Native to Bolivia, few information

Table 1 (continued)

Section		Botanical characteristics	Species	Vernacular name	Main information
			Juglans steyermarkii Mann.	Guatemalan walnut	Only found in Huehuetenango department of Guatemala, can reach 17 m high, few information
			Juglans venezuelensis Mann.	Venezuelan walnut	Native to Venezuela, small tree, 2 known wild populations of less than 100 individuals, few information
White walnut	Trachycaryon	Distinguished from black walnut by a more pubescent and viscous husk, nut shell does not have secondary wall, nuts are in clusters	*Juglans cinerea* L.	Butternut	Native to Northeastern USA, near to Canada, spontaneous until Minnesota and Arkansas, spreading shrub up to 20 m high, light gray bark and very cracked when mature, susceptible to sunburn, wood of lower grade than common and black walnut, very cropper, early budding, poor compatibility with *J. regia*, lengthened nut with thick shell, 11/17 lanceolate and dentate leaflets
–	Cardiocaryon	From the Far East, like *J. cinerea*: nuts in clusters without secondary wall, in pubescent and viscous husk but not dehiscent. Distinguished from *J. cinerea* by leaflets sparsely dentate and cordate leaf scar whose upper edge is concave	*Juglans sieboldiana* Maxim. (*J. ailantifolia* Carr.)	Japanese walnut	Native to Japan where it is abundant in forests, 15/20 m high, light gray bark, poor compatibility with *J. regia*, very cropper, early budding, nuts in cluster of 12/20, ovoid shape, pointed top, smooth shell, thick primary wall with lacunae, 11/17 oblong leaflets and few dentate, pubescent on lower side
			Juglans cathayensis Dode	Chinese walnut	Native to Central and Eastern China, spreading shrub up to 25 m high, used as rootstock in China, 9/17 acuminate leaflets, pubescent on lower side with glandulous petiole, nuts in cluster of 6/10, sherical to ovoid shape, thick smooth shell
			Juglans mandshurica Maxim.	Manchurian walnut	Native to Manchuria and Korea, found in Amur river valley, resistant to extreme cold, used as rootstock in cold areas of Northern China, spreading shrub, light gray bark, 30 m high, used for its wood, leaves until 90 cm, 9/17 oblong and acuminate leaflets, few dentate, pubescent on lower side, distinguished from *J. sieboldiana* by nuts in small cluster of 5/7, rough and thick shell

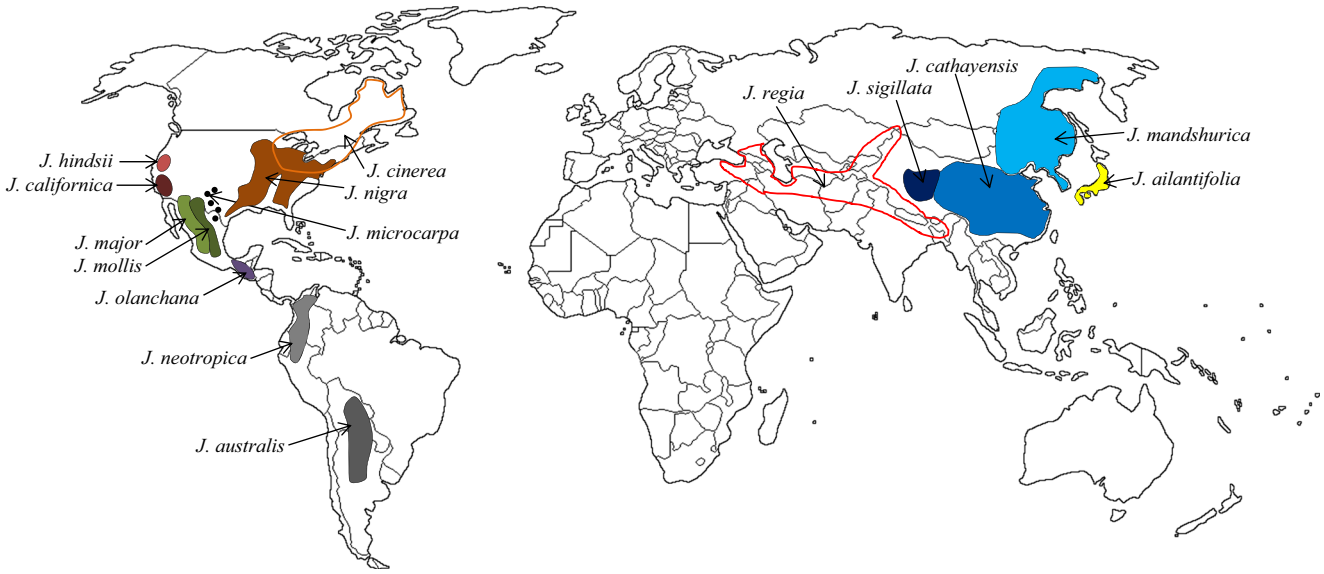

Fig. 2 Simplified distribution of *Juglans* taxa adapted from Aradhya et al. (2007) and Germain et al. (1999). Distribution of cultivated species *J. regia* extends beyond its natural range

eastern region of Turkey (Dode 1909; Forde 1975) and reached east into Northern India, Nepal, and Western China where it is still possible to find wild forests (Leslie and McGranahan 1988). Although Persian walnut was introduced into Europe and the Balkans before the last glacial period (~50.000 ya) (Carrion and Sanchez-Gomez 1992), it is still debated how trees have reached Europe. In relation to that, a very recent study focused on the history of its spread across Europe has integrated various data such as fossil pollen and microsatellite markers (Pollegioni et al. 2017) and came to the conclusion that natural populations existed before human intervention. This piece of work also confirmed the presence of walnut in glacial refugia in Western Europe and in the Balkans. This is why it would appear that human-mediated dispersal between Asia and the Balkans started in the Early Bronze Age. Another recent study confirmed this hypothesis and in addition showed that the highest genetic diversity of Persian walnut is found in South Asia, suggesting that this region is probably the origin of Persian walnut (Roor et al. 2017). Alternatively, Cro-Magnon people could have eaten ancestral forms of Persian walnuts (~17.000 ya) as archeological evidence found in the Perigord French region suggests. During the time of Ancient Greece, Persian walnuts became dispersed and more abundant thanks to Alexander the Great (~350 B.C.) and increased human dispersal of walnuts into Portugal; Spain and North Africa took place during the Roman Empire (~27 B.C. to ~395 A.D.). Evidence suggests human-mediated dispersal during the Roman Empire between Western Europe and the Balkans in Eastern Europe (Pollegioni et al. 2017). In the meantime, Chinese archives suggest a form of domestication of walnut in Southern Tibetan and the Yunnan region during the Han dynasty (~206 B.C. to ~220 A.D.) (De Candolle 1885). In the sixteenth century, the conquest of the New World enabled the spread of Persian walnut into South America, particularly Chile, before introduction in its present form to California in the nineteenth century (Germain et al. 1999).

Biology, phenology, and physiology

As described by Germain et al. (1999), walnut trees are wind-pollinated monoecious and dichogamous plants. Walnut trees are self-compatible but self-fertilization is limited due to the dichogamy, so it is often necessary to include a pollinizer genotype in orchards. Male inflorescences each consist of 100 to 160 flowers formed into a catkin that can produce around two million pollen grains. Female inflorescences of most walnut species, including Persian walnut, develop as individual flowers or most commonly as two or three per inflorescence. Female flowers of species in section *Cardiocaryon* are borne in racemes of approximately 7–19 flowers each. There are two main types of fruiting: fruiting only at the terminal position on new branches or fruiting at both terminal and lateral positions on shoots (fruiting all along branches). Rather rare, this last fruiting type was originally introduced into California by the cultivar "Payne." Walnut fruit consists of a fleshy green husk surrounding a nucleus of the walnut itself, composed of a shell containing the kernel.

There is evidence that tree phenology responds to climate changes: earlier spring budburst and later autumn senescence have extended the growing season by approximately 11 days since the 1960s at mid to high latitudes (Menzel and Fabian 1999). However, trees need to have completed a series of processes before the first frost arrives or they risk damage (Way 2011). In walnut, buds enter dormancy from mid-

August to early October and under temperate climate conditions; chilling requirements necessary for breaking dormancy are normally met by early January (Germain et al. 1999). Budburst dates vary according to genotype and climatic conditions, but under French climate conditions, leafing occurs between mid-March and early June. Fruit maturity typically occurs in September and October (Germain et al. 1999). A study of walnut phenology, conducted for two winters at both lowland and mountain locations, found no significant differences in chilling requirements among genotypes differing in budburst dates, but heat requirements were significantly different (Charrier et al. 2011). This means that from September until January, the acclimation was driven mainly by environmental factors, but from January until budburst, a prevalent genotype effect was found.

Production and nutritional aspects

Among the nut crops, almonds had the greatest production until 2008, then the walnut took over until 2013 (Fig. 3). During the 1973–2014 period, the five largest walnut producers were China, the USA, Iran, Turkey, and Ukraine with at the leading front the USA between 1973 and 1983, and China nowadays. In 2014, in-shell walnut production was as follows: (1) China, 1602 kt; (2) The USA, 518 kt; (3) Iran, 446 kt; (4) Turkey, 181 kt; (5) Mexico, 126 kt. France was in 10th position in 2014 with 34 kt. Regarding nutritional aspects, walnut is highly caloric with 654 kcal per 100 g and with high amount of proteins (15.2 g), lipids (65.2 g), carbohydrates (13.7 g), and micronutrients (US Department of Agriculture, Agricultural Research Service, Nutrient Data Laboratory 2015). Walnuts are particularly rich in unsaturated fatty acids, including oleic, linoleic, and linolenic acids (Maguire et al. 2004).

A study published by Martínez et al. (2010) focused on the biochemistry of walnut and highlights the nutritional importance of the oil made up of tocopherols, phospholipids, sphingolipids, sterols, and phenolic compounds. In addition, antioxidant and anticancer activities of walnut protein hydrolysates show possible benefits for prevention of breast and colon cancer (Jahanbani et al. 2016). Another recent review focused on the phytochemical profiles and biological activity of *J. regia* components and discusses the current high interest in isolation and identification of these active compounds and screening them for pharmacological activities (Panth et al. 2016). Concerning the effects on blood lipids, walnut consumption led to a decrease in low-density lipoprotein (LDL) cholesterol with no change in high-density lipoprotein cholesterol (Banel and Hu 2009). Taha and Al-Wadaan (2011) also reviewed the activities of walnut constituents, and similar observations have been published for tree nuts in general (Bolling et al. 2010).

Morphological studies as preliminary breeding approaches

Plant breeding has always impacted food production and played a vital role in improving human nutrition (Tester and Langridge 2010). However, this has also increased uniformity within the world's agricultural crops, contributing to increased genetic vulnerability to biotic and abiotic stresses (Keneni et al. 2012). For these reasons, it is important to understand better the impacts of modern plant breeding on genetic diversity. In the same way, intelligent management of this diversity could be of valuable assistance to breeders. Germplasm diversity is commonly evaluated with the help of morphological descriptors. This is usually the first step in classifying and describing germplasm and in studying heritability of traits for a new breeding program and selection of superior genotypes (Smith and Smith 1989). For this purpose, the International Union for the Protection of New Varieties of Plants (UPOV) offers guidelines in determining distinctness, homogeneity, and stability of new cultivars for most plants. Thus, morphological studies provide direction for the choice

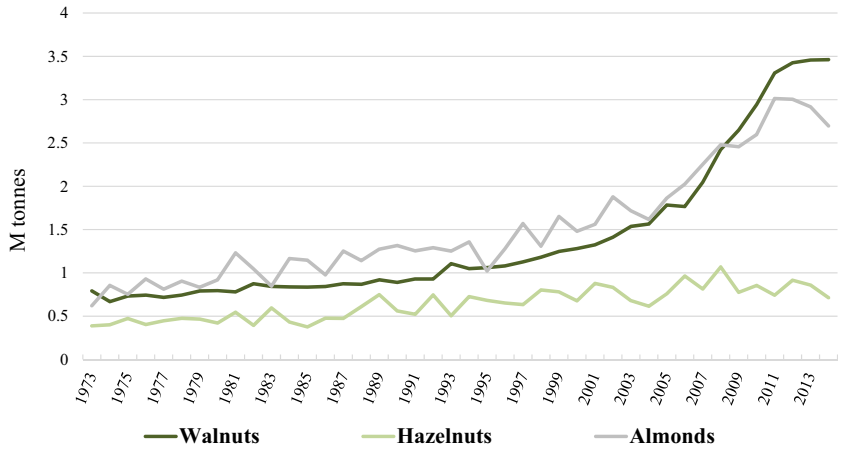

Fig. 3 World in-shell production of walnuts, hazelnuts, and almonds between 1973 and 2014 (FAOSTAT data 2016)

of cultivars suited to specific growing conditions (Solar and Stampar 2011).

For *J. regia*, many early morphological studies concerned the search of correlation between various horticultural traits (Sholokhov 1974; Komanich 1980; Sen 1985; Sharma 1996). While the relationship between yield level and type of dichogamy was found conflicting (Kornienko 1974; Majacka 1971), another study found no statistical difference between protogynous and protandrous cultivars on average yield and nut characteristics (Akça and Sen 1997). Then, a correlation was found between kernel weight and flowering type (Eskandari et al. 2005). Moreover, Amiri et al. (2010) found a strong correlation between lateral-bearing habit and yield in Iranian genotypes. However, lateral-bearing trees observed in Slovenia were reported to be more susceptible to walnut blight and cold (Solar et al. 2001) and susceptibility to walnut blight correlated with leaf abscission date (Abedi and Parvaneh 2016). Moreover, a negative correlation between yield and tree height was reported by Forde and McGranahan (1996), and a positive correlation was found between altitude and frost resistance, although altitude correlated negatively with yield (Atefi 1990).

All these traits are influenced by three components: genetic background, environment, and interaction between the two; it is of utmost importance to decipher the amount of contribution of each of these components. In Iran, an important study focused on the heritability of horticultural traits such as leafing and flowering times, fruit and kernel weights, kernel color, shell texture, etc. Broad-sense heritabilities (H^2) were higher than 60% among the genotypes tested, except for spring frost susceptibility and kernel percentage (Eskandari et al. 2005). A previous piece of work already focused on the narrow-sense heritabilities (h^2) for some traits and found many were high: leafing date 96%, female and male blooming dates > 80%, harvest date 85%, and fruit traits (shell thickness, nut and kernel weights) 85%. Conversely, yield and kernel color seemed to be non-heritable (Hansche et al. 1972).

Morphological studies can greatly assist programs in countries, such as Iran, using selection from existing natural seedling populations (Ghasemi et al. 2012). Promising genotypes there were selected by their phenological and pomological traits, including leafing date, lateral bearing, type of dichogamy (Ghasemi et al. 2012; Ebrahimi et al. 2015), bud break (Arzani et al. 2008), spring frost tolerance (Mahmoodi et al. 2016), nut yield, nut maturity, and nut characteristics (Haghjooyan et al. 2005; Arzani et al. 2008; Ahandani et al. 2014; Khadivi-Khub et al. 2015). Moreover, vigor of dwarf seedlings, which can be used to increase planting density, was also studied in Iran (Rezaee et al. 2009). A positive correlation was found between seedling height and other parameters, including seedling diameter, number of nodes, and length of internodes, suggesting that measurement of seedling height alone is sufficient for identification of dwarf genotypes.

Similar studies were performed in Turkey (Bayazit 2012). Superior genotypes were selected for yield, nut characteristics, and cold tolerance (Aslantaş 2006) and other promising trees were selected for late flowering and walnut blight and anthracnose tolerance (Yarilgac et al. 2001; Asma 2012; Fikret Balta et al. 2007; Karadag and Akça 2011). In Albania, evaluation of pomological and phenological traits of germplasm from the Dibra region found high variability in nut weight (Zeneli et al. 2005). In additional studies of this type, walnut cultivars were evaluated in Serbia (Miletic et al. 2003) as well as some foreign cultivars under the climatic conditions of south Bulgaria (Gandev and Dzhuvinov 2015). In India, differences between geographic and genetic diversity were identified using 15 nut traits (Sharma and Sharma 2001). In Italy, a study of a simulated seed orchard, planted with genotypes selected by morphology and phenology in order to preserve genetic resources, showed that it could have as much variability as a larger population (Ducci et al. 2010). In France, 2450 hybrids from 22 crosses between French and California varieties were studied. A correlation was found between leafing date of 1-year-old trees and the same parameter some years later on the same trees, showing that an early screening of this trait is possible (Germain 1990). Finally, the rate of apomixis was also investigated and variation among numerous European cultivars in Mediterranean climate was recorded (Asadian and Pieber 2005).

Historical overview of molecular marker development and use

Phenotypical studies are essential for a general description of an organism, and have been used since Darwin's time due to their low cost and wide application, for example for museum and fossil specimens. However, the arrival of molecular investigation methods in plant science research gives new insights in both selection processes and evolutionary studies. Indeed, when individuals are morphologically similar or indistinguishable, molecular studies can provide information based on their DNA and heritable variation (Hillis 1987). Molecular information seems less affected by environment than are morphological data, which often depict conflicting scenarios (Hillis 1987). On top of this, morphological markers can affect one another and make difficult to predict pleiotropic effects (Andersen and Lübberstedt 2003). As a consequence, nowadays, there is a debate on the importance and the future of morphological studies, especially in the context of phylogenetic systematics (Jenner 2004). Application of a wide range of molecular markers has been the focus of many walnut studies during the past 30 years. The following sections depict an overview of these molecular studies and their application (Table 2).

Table 2 Applications of molecular markers in walnut studies

Molecular markers	Applications	References
Isozymes	Inheritance evaluation	Arulsekar et al. 1986
	Intergeneric hybrids identification	McGranahan et al. 1986
	Genotype origin determination	Cheng and Yang 1987
	Genetic diversity/relationships determination	Malvolti et al. 1993, 1994; Fornari et al. 2001; Busov et al. 2002; Ninot and Aletà 2003
	Genotype characterization	Aletà et al. 1990; Solar et al. 1994; Vyas et al. 2003
isozymes + RFLPs	Somatic embryo origin determination	Aly et al. 1992
RFLPs	Genetic diversity/relationships determination	Fjellstrom et al. 1994; Fjellstrom and Parfitt 1994b
	Genetic mapping	Fjellstrom and Parfitt 1994a
RFLPs + RAPDs	Introgression evaluation	Woeste et al. 1998
	Genetic mapping	Woeste et al. 1996b
RAPDs	Interspecific hybrids identification	Ross-Davis et al. 2008; Emilia et al. 1997
	Biotic stress resistance	Woeste et al. 1996a
	Phenology/pomology evaluation	Keqiang et al. 2002; Li et al. 2007
	Genetic diversity/relationships determination	Nicese et al. 1998; Fatahi et al. 2010; Qianwen et al. 2010; Pop et al. 2010; Erturk and Dalkilic 2011
	Genetic mapping	Malvolti et al. 2001
ISSRs	Genotype origin determination	Malvolti et al. 2010
	Genetic diversity/relationships determination	Potter et al. 2002; Christopoulos et al. 2010; Ji et al. 2014; Pollegioni et al. 2003
AFLPs	Homogamous/dichogamous genetic diversity comparison	Sütyemez 2006
	Biotic stress investigation	Loreti et al. 2001; Mozaffarian et al. 2008
	Genetic diversity/relationships determination	Kafkas et al. 2005; Bayazit et al. 2007; Chen et al. 2008; Chen et al. 2009; Qing Guo et al. 2010; Wang et al. 2010; Ma et al. 2011; Ali et al. 2016
	Genotype characterization	He et al. 2010
AFLPs + RAPDs	Genetic diversity/relationships determination	Xu et al. 2012b
SSRs	Markers discovery	Woeste et al. 2002; Dangl et al. 2005; Hoban et al. 2008; Zhang et al. 2010; Qi et al. 2011; Yi et al. 2011; Wu et al. 2012; Zhang et al. 2013; Chen et al. 2013; Najafi et al. 2014; Topçu et al. 2015; Ikhsan et al. 2016; Dang et al. 2015; Hu et al. 2015; Dang et al. 2016
	Interspecific hybrids identification	Pollegioni et al. 2008, 2009
	Genetic diversity/relationships determination	Foroni et al. 2005; Dangl et al. 2005; Victory et al. 2006; Robichaud et al. 2006; Wang et al. 2008; Aradhya et al. 2009; Gunn et al. 2010; Karimi et al. 2010; Bai et al. 2010; Mohsenipoor et al. 2010; Ebrahimi et al. 2011; Ruiz-Garcia et al. 2011; Pollegioni et al. 2011; Qi et al. 2011; Grauke et al. 2012; Mahmoodi et al. 2013; Zhang et al. 2013; Pop et al. 2013; Najafi et al. 2014; Dang et al. 2015; Hu et al. 2015; Han et al. 2016; Noor Shah et al. 2016; Ćelepirović et al. 2016; Wang et al. 2016; Vischi et al. 2017; Ebrahimi et al. 2017
	Origin/history	Pollegioni et al. 2017; Roor et al. 2017
	Genotype characterization	Foroni et al. 2007
SNPs	Markers discovery	Liao et al. 2014
	Biotic stress resistance	Zhu et al. 2015
	Biotic stress investigation	Zerillo et al. 2014
	Synteny analysis	Luo et al. 2015
	Genotype characterization	Ciarmiello et al. 2011
	Genetic mapping	You et al. 2012

From isozyme markers in late 1980s to AFLPs markers in early 2000s

In the late 1980s, knowledge of fruit trees at the genetic level was poor but a major breakthrough was the development of enzyme-based markers using isozyme electrophoresis (Hunter and Markert 1957). Identification and characterization of isozymic genes in *J. regia*, such as that of phosphoglucomutase, was first reported by Arulsekar et al. (1986). Then, intergeneric hybrids between *Pterocarya* spp. and *J. regia* were identified based on isozyme variation (McGranahan et al. 1986) and the origin of different walnut types in China and Tibet was examined (Cheng and Yang 1987). Moreover, genetic diversity in Italian *J. regia* populations was reported (Malvolti et al. 1993) and in Slovenia, a study showed that pollen enzymes have more variability than those of leaves (Solar et al. 1994). Furthermore, a study of leaf and fruit morphology and isozyme variation in populations of *J. regia* from central Italy showed an exclusive correlation between genotype and leaf morphology and between leaf morphology and fruit morphology. This suggests a chain of causal relationships between genotype and the two studied aspects of phenotype (Malvolti et al. 1994). Other studies have used isozymes to analyze genetic diversity/relationships in *J. regia* (Fornari et al. 2001; Ninot and Aletà 2003), *J. nigra* (Busov et al. 2002), or for cultivar identification (Aletà et al. 1990; Vyas et al. 2003).

Molecular genotyping constitutes a valuable efficient method to screen germplasm collections and facilitate the use of these resources by breeders and researchers (Mason et al. 2015). The emergence of DNA manipulation techniques drove the shift from enzyme-based to DNA-based markers in the early 1990s (Schlötterer 2004). DNA markers are less limited in number, and there is no need to wait for a particular development stage of the plant, making early scoring possible (Andersen and Lübberstedt 2003). In *J. regia*, a study combining isozyme and restriction fragment length polymorphism markers (RFLPs) (Botstein et al. 1980) was performed in order to determine the origin (zygotic or maternal) of somatic embryos derived from ovule tissues (Aly et al. 1992). It led to a fast parentage determination of plants regenerated in vitro. Then, a California team conducted three studies in 1994 using RFLPs. The first used unweighted pair group method with arithmetic mean (UPGMA) cluster analysis (Nei 1972) to highlight that California germplasm was associated with that of France and Iran and had less similarity to that of Nepal, China, and Japan (Fjellstrom et al. 1994). A second study focused on analysis of an interspecific backcross [*J. hindsii* × *J. regia*] × *J. regia* created in order to construct the first linkage map in walnut, which included 12 linkage groups (LGs) covering 1660 cM (Fjellstrom and Parfitt 1994a). The third study concentrated on determination of taxonomic relationships between different *Juglans* species (Fjellstrom and Parfitt 1994b) and indicated that *J. cinerea* should be included in *Cardiocaryon* section and not as the unique section *Trachycaryon*, as suggested in existing literature (Manning 1978).

The main limitations of the RFLP technique are the low level of polymorphism, the complexity of its use (Wani et al. 2010), the lack of automation, and the large amount of pure DNA needed (Schlötterer 2004). Hence, new strategies based on polymerase chain reaction (PCR) were developed and among these were randomly amplified polymorphic DNAs (RAPDs) (Williams et al. 1990) and inter-simple sequence repeats (ISSRs) (Zietkiewicz et al. 1994). A California team identified RAPDs in a backcross population [*J. hindsii* × *J. regia*] × *J. regia* and created a revised genetic map of walnut that also included the set of RFLPs published earlier by Fjellstrom and Parfitt (1994a). This work expanded the number of identified LGs from 12 to 15, still short of the 16 total LGs present in walnut (Woeste et al. 1996b). Another study by this team identified and characterized an RAPD marker in walnut linked to the hypersensitivity response to cherry leafroll virus, which causes walnut blackline disease, a fatal necrosis of the graft union (Woeste et al. 1996a). Examination of another walnut backcross population {[*J. hindsii* × *J. regia*] × *J. regia*} found a low correlation between genetic and morphological distance during introgression (Woeste et al. 1998), suggesting that morphology-based selection during introgression is not an effective method for recovering the parent genome. RAPD markers also were used to study the genetic relatedness among *J. regia* cultivars in the collection of the breeding program at the University of California, including "Chandler," "Payne," "Meylan," and "Franquette" (Nicese et al. 1998). This led to the identification of two separate groups: (1) Asian genotypes and (2) California and French cultivars. Then, an additional linkage map was created using RAPDs and a *J. regia* intraspecific cross but the mapping efficiency was lower than with the interspecific cross (Malvolti et al. 2001). Phenological and pomological traits were also investigated using RAPD analysis. One marker was found to be related to early bearing trait (Keqiang et al. 2002), and one sequence-characterized amplified region (SCAR) marker derived from an RAPD was correlated with shell thickness (Li et al. 2007). In Italy, ISSRs were used successfully to differentiate varieties coming from the north or the south of the country (Pollegioni et al. 2003). An additional study was carried out using *Juglans* × *quadrangulata* (Carr.) Rehd. (pro sp.), a hybrid between *J. cinerea* (butternut) and *J. regia* (Ross-Davis et al. 2008). These hybrids are vigorous and more resistant to butternut canker disease, and RAPDs differentiating pure butternut from hybrids were found. Hybrids between *J. nigra* and *J. regia* were also differentiated using RAPDs (Emilia et al. 1997). RAPDs and ISSRs have been used since by Iranian (Fatahi et al. 2010), Romanian (Pop et al. 2010), Chinese (Qianwen et al. 2010;

Ji et al. 2014), Greek (Christopoulos et al. 2010), and Turkish (Erturk and Dalkilic 2011) teams to characterize their own local *J. regia* germplasm. In addition, the genetic relationships among *J. regia* cultivars from California, Europe, and Asia were examined using ISSRs but results showed that the ISSR method is limited for the study of germplasm coming from different geographic regions (Potter et al. 2002). Finally, a multidisciplinary approach was tried in Italy by combining the use of ISSRs, agronomic measures on fruit (diameter, form, dry weight), and chemical composition of cultivars from different areas, in order to determine genotype provenance. ISSRs showed a strong ability to discriminate walnut provenances, and the genotyping results were partly confirmed by the morphological and biochemical analysis of fruits (Malvolti et al. 2010).

Amplified fragment length polymorphism markers (AFLPs) (Vos et al. 1995) have been used in a number of studies. AFLPs were used to investigate the genetic diversity of the causal agent of walnut blight *Xanthomonas arboricola* pv. *juglandis*, one of the most important diseases of *J. regia* (Loreti et al. 2001). In this analysis, 66 isolates of *X. arboricola* pv. *juglandis* from different countries were characterized. AFLPs were also used to examine variation in populations of host-associated carob moth (*Ectomyelois ceratoniae*) on pomegranate, pistachio, and walnut in Iran (Mozaffarian et al. 2008). In addition, AFLPs were used in determining that the genetic diversity is higher in homogamous than in dichogamous genotypes, due to a higher level of heterozygosity (Sütyemez 2006). Then, Turkish *J. regia* trees were characterized using AFLPs in order to select superior genotypes (Kafkas et al. 2005) and AFLPs were used to study the genetic diversity among Turkish genotypes with a low chill requirement (Bayazit et al. 2007). In China, wild *J. regia* and *J. sigillata* populations from Sichuan province were studied using AFLPs to understand the genetic relationships between these two economically important species in the province (Chen et al. 2008, 2009). Other Chinese teams used AFLPs to fingerprint walnut cultivars (He et al. 2010; Ma et al. 2011; Xu et al. 2012b) and to study the genetic diversity of the early fruiting trait (Qing Guo et al. 2010). Eight *Juglans* species and one genotype of *Pterocarya stenoptera* were also studied in China with AFLPs to understand their genetic relationships (Wang et al. 2010). Finally, five walnut populations from the Kurdistan region of Iraq were analyzed using AFLPs and their genetic diversity was found to be low (Ali et al. 2016).

Peak of molecular studies with SSRs from the late 2000s

Simple sequence repeats (SSRs) (Morgante and Olivieri 1993), also known as microsatellites, are very powerful and informative markers for fingerprinting genotypes and determining genetic relationships, due to their abundance, co-dominant status, high reproducibility (Wani et al. 2010), and high polymorphism (Singh et al. 2008).

SSR markers have been employed for numerous walnut studies (Table 3). The first walnut SSR study was performed on *J. nigra* accessions to fingerprint clones with high-quality timber and edible nuts (Woeste et al. 2002). Two additional studies using SSRs on *J. nigra* showed a low genetic diversity among several populations from the central hardwood region of the USA (Victory et al. 2006) and identified parentage among progenies of several families (Robichaud et al. 2006). Another study characterized *J. nigra*, *J. regia*, and natural hybrids between the two species *Juglans* × *intermedia* (Carr.) with SSR markers (Pollegioni et al. 2008). Identification of parental species was found possible in a mixed Italian population, and this work confirmed the transferability of *J. nigra* SSRs to *J. regia*. A similar study focused on identification of parents that can easily hybridize for production of hybrid progeny, especially those characterized by increased vigor and a better wood quality (Pollegioni et al. 2009).

In China, genetic diversity of *J. regia* and *J. sigillata* populations, the two main cultivated species, was assessed using *J. nigra* SSR markers and a UPGMA dendrogram showed a geographic gradient rather than the conventional taxonomic classification (Wang et al. 2008). The results suggest that the genetic relatedness between the two species is strong, information relevant to determining if they are different species or merely different ecotypes. Moreover, a different study of the genetic diversity of *J. regia* and *J. sigillata* also using SSRs was directed at understanding the role of human impacts and biodiversity of walnut in Yunnan area in China (Gunn et al. 2010). The marker data showed that the two species were indistinguishable, and the authors concluded with two scenarios: *J. regia* and *J. sigillata* could be incipient species or they are distinct species that exchange genes. Next, expressed sequence tag (EST)-SSRs were developed and used to study trees of *Juglans* sections *Rhysocaryon*, *Cardiocaryon*, *Dioscaryon*, and *Carya illinoinensis* coming from the USA and Asia (Qi et al. 2011). The resulting UPGMA dendrogram clustered the samples according to conventional taxonomy, with *J. regia* and *J. sigillata* well separated, and proved the good transferability of EST-SSRs among *Juglans* species.

Other *Juglans* species, such as natural populations of *J. mandshurica* in northern and northeastern China, were also analyzed using SSRs (Bai et al. 2010). The results, which also included cpDNA fragments, showed that two independent refugia were maintained in northern China during the last glacial period, contrary to numerous other temperate forest trees which migrated to southern China. Then, newly developed ESTs from *J. regia* were used to develop SSR primers for *J. nigra* and *Carya* spp. investigation and many of these markers were found to be transferable with a high level of

Table 3 Microsatellite marker development in walnut

Authors and year	Generation source	*Juglans* species	Number of markers	Markers name
Woeste et al. 2002	Enriched (GA/CT)n SSR library from genomic DNA	*J. nigra*	30	WGA002, 004, 006, 007, 011, 017, 024, 025, 027, 032, 033, 042, 045, 047, 053, 054. 056, 058, 060, 065, 069, 070, 071, 072, 073, 074, 078, 079, 080, 082
Dangl et al. 2005	Enriched (GA/CT)n SSR library from genomic DNA	*J. nigra*	12	WGA001, 009, 089, 118, 202, 225, 276, 321, 331, 332, 349, 376
Hoban et al. 2008	Enriched (CA/GA/TAG)n SSR library from genomic DNA	*J cinerea*	13	jcin_A5, B110, B112, B114, B12, B121, B147, B157, B159, B212, B249, B262, B264
Zhang et al. 2010	5025 EST sequences from the NCBI database	*J. regia*	41	Contig_5, 40, 62, 104, 156, 216, 259, 268, 269, 352, 407, 541, 562-2, 566, 610, 642, 718, 719, 721, 795, 870, 1000, 1172, 1396, 1458, 1464, 1483, 1528, 1529, 1552, 1625, 1626, 1631, 1632, 1656, 1681, 1682, 1687, 1692, 1693, 1712
Qi et al. 2011	5025 EST sequences from the NCBI database	*J. regia*	18	ZMZ3, 5, 6, 11, 13, 14, 22, 26, 27, 30, 31, 34, 35, 44, 45, 46, and ZY1, 16
Yi et al. 2011	5213 EST sequences from the NCBI database	*J. regia*	30	/
Wu et al. 2012	48,218 BAC-end sequences from the NCBI database	*J. regia*	13,675	Mailable for downloading at http://walnutgenome.ucdavis.edu/
Zhang et al. 2013	13,559 EST sequences from the NCBI database	*J. hindsii* x *J. regia*	76	BFU-Jr10, 19, 29, 38, 40, 41, 42, 45, 46, 48, 49, 52, 62, 67, 68, 69, 70, 73, 82, 95, 97, 98, 99, 102, 104, 116, 121, 126, 130, 132, 144, 150, 152, 154, 161, 163, 167, 171, 179, 181, 182, 183, 184, 185, 195, 196, 201, 207, 217, 219, 228, 230, 233, 238, 240, 243, 245, 247, 249, 250, 254, 256, 263, 275, 276, 277, 284, 286, 288, 292, 298, 301, 306, 307, 319, 321
Chen et al. 2013	Fast isolation by AFLP of sequences containing repeats (FIASCO) approach	*J. mandshurica*	20	Pl, 3, 5, 8, 12, 14, 16, 18, 21, 23, 26, 28, 29, 30, 31, 35, 41, 44, 45, 47
Najafi et al. 2014	Fast isolation by AFLP of sequences containing repeats (FIASCO) approach	*J. regia*	13	ABRII/Jr1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13
Topçu et al. 2015	Enriched (CA/GA/AAC/AAG)n SSR library from genomic DNA	*J. regia*	185	46 SSRs from CA enriched library named CUJRAxxx, 80 SSRs from GA enriched library named CUJRBxxx, 14 SSRs from AAC enriched library named CUJRCxxx, and 45 SSRs from AAG enriched library named CUJRDxxx
Dang et al. 2015	Next-generation sequencing of transcriptome	*J. cathayensis*	12	JC6576, 9827, 4088, 4535, 3412, 2995, 7329, 5411, 8125, 1151, 3304, 4833
Hu et al. 2015	Next-generation sequencing of transcriptome	*J. hopeiensis*	25	JH6514, 2678, 9978, 2753, 2751, 8883, 1908, 6876, 6044, 0405, 8061, 1424, 9127, 9543, 2096, 4093, 4548, 1195, 0608, 8168, 0190, 2576, 9664, 4114, 5908
Ikhsan et al. 2016	48,218 BAC-end sequences from the NCBI database	*J. regia*	307	/
Dang et al. 2016	Next-generation sequencing of transcriptome	*J. regia*	39	JR0082, 0160, 1165, 1739, 1817, 2018, 2465, 2510, 2600, 2873, 3147, 3434, 3608, 3773, 4051, 4324, 4616, 4964, 4965, 4965.2, 5538, 5574, 6160, 6226, 6439, 6508, 6638, 6714, 6742, 6926, 7171, 7363, 7495, 7495.2, 7544, 8058, 8815, 9306, 9632

polymorphism (Zhang et al. 2013). Finally, *J. nigra*, *J. major*, and *J. microcarpa* individuals from natural population from Texas were investigated using plastid SSR markers suggesting that interspecific hybridization has occurred within these species. Indeed, many accessions considered to be *J. major* were hybrids with *J. microcarpa* (Grauke et al. 2012).

In *J. regia*, the most important Italian landrace "Sorrento" was fingerprinted with SSR markers (Foroni et al. 2005, 2007), as well as other Italian walnut resources, to discover and characterize long-term human impacts and frequent human-mediated domestication events controlling genetic diversity (Pollegioni et al. 2011). An analysis of the genetic structure in *J. regia* using SSRs revealed a high level of genetic diversity with five genetic groups linked to the centers of diversity of walnut in Eurasia (Aradhya et al. 2009). Later, SSR markers were also used in Iran to study the genetic diversity of genotypes (Karimi et al. 2010; Mohsenipoor et al. 2010; Ebrahimi et al. 2011; Mahmoodi et al. 2013; Najafi et al. 2014). Cultivars, mainly from Spain and the USA, were studied with SSR markers and data separated Spanish cultivars from those of California (Ruiz-Garcia et al. 2011). Additional studies using SSRs have focused on germplasm diversity and genetic relationships among Indian genotypes (Noor Shah et al. 2016), among trees from cold-temperate areas of the USA and Europe (Ebrahimi et al. 2017), and among germplasm in China (Han et al. 2016; Wang et al. 2016) and Romania (Pop et al. 2013). Finally, two recent papers have been published. The first focused on optimization of high-throughput multiplex PCR by combining 11 SSRs, particularly from the two first set of markers developed in *J. nigra*, in order to investigate the genetic diversity of Croatian germplasm (Ćelepirović et al. 2016). The other concerns natural populations of *J. regia* in the eastern Italian Alps where moderate genetic diversity was found (Vischi et al. 2017). These authors also claim that some SSR markers reported in the literature, especially from the two first set of *J. nigra* markers, were unable to provide reliable results with a lack of polymorphism, multilocus amplifications, and high frequency of null alleles.

Additional sets of SSR markers were developed in *J. regia* from bacterial artificial chromosome (BAC) end sequences (Wu et al. 2012; Ikhsan et al. 2016), from DNA genomic libraries with specific pattern repeats such as "AAC" or "AAG," improving the number of informative alleles (Topçu et al. 2015), and with EST sequences from the transcriptomes of many organs (Dang et al. 2016). These could potentially be useful in germplasm characterization and linkage mapping.

SNPs revolution from the early 2010s

Single nucleotide polymorphism (SNP) markers (Wang et al. 1998) present many advantages compared to the previous markers as they are highly abundant and are now the markers of choice for automated high-throughput analysis at moderate cost (Schlötterer 2004).

The initial use of SNP markers to characterize *J. regia* cultivars (Ciarmiello et al. 2011) employed the first and second internal transcribed spacer regions (ITS1 and ITS2) and 5.8S coding region of rDNA-repeat (ribosomal DNA) operon segment. Alignment of sequences revealed 244 SNPs and one short insertion-deletion (InDel). The phylogenetic analysis showed that these regions could be used for specific authentication of *J. regia* cultivars. Moreover, the development of a pipeline was completed in order to discover genome-wide SNPs in coding sequences. Twenty-two thousand seven hundred ninety-nine SNPs in "Chandler" were identified and 6000 SNPs were selected to construct a chip to be used to genotype a mapping population (You et al. 2012). Then, by partial genome pyrosequencing, 48,165 SNPs and 1037 InDels were found in *J. regia* (Liao et al. 2014). These sets of SNPs could provide great assistance for genetic diversity analysis, for understanding nucleotide variations in genome, for genetic map construction, and for other breeding applications such as marker-assisted selection. The causal agent of thousand cankers disease in the USA, *Geosmithia morbida*, was also investigated using SNP analysis of 209 isolates in a population genetic study (Zerillo et al. 2014).

Finally, analysis with SNPs assessed synteny between walnut and several other plant species. These included three long-lived perennials (*Vitis vinifera*, *Populus trichocarpa*, and *Malus domestica*) and three short-lived herbs (*Cucumis sativus*, *Medicago truncatula*, and *Fragaria vesca*). Results showed that the walnut genome was closer to the three genomes of woody perennials than to those of the herbs, and that degree of synteny was linked to nucleotide substitution rates (Luo et al. 2015).

Genetic/physical mapping and QTL detection

Genetic maps (or linkage maps) indicate the position and relative genetic distances between markers along chromosomes, based on the principle that markers segregate during meiosis as a result of chromosome recombination (Paterson 1996). Genetic mapping is the first step in localization of genes or quantative trait loci (QTLs) associated with agronomic traits of interest. Genetic maps are desirable for breeding and useful in marker-assisted selection (MAS) (Semagn et al. 2006). A number of intraspecific and interspecific populations have been developed in walnut for genetic mapping construction and QTL detection (Table 4).

First genetic maps with RFLPs and RAPDs

The first genetic map in walnut was created with the use of RFLP markers. A progeny of 63 individuals from an

Table 4 Summary of genetic linkage maps for walnut

Species/country	Mapping population type	No./types of offspring	No./types of markers	No. of LG/genome size (cM)	References
[*J. hindsii* × *J. regia*] × *J. regia* /USA	"Paradox Mom" × "Hartley"	63/BC1	42 RFLP	12/1660	Fjellstrom and Parfitt 1994a
[*J. hindsii* × *J. regia*] × *J. regia* /USA	"Paradox Mom" × "Hartley"	49/BC1	59 RAPD, 48 RFLP	15/NA	Woeste et al. 1996b
J. regia/Italy	"Lara 480" × "Chandler 1036"	81/F1	120 RAPD, 4 isozymes	11 (female), 10 (male)/NA	Malvolti et al. 2001
J. regia/China	"Chandler" × "Idaho"	425/F1	1525 SNP	16/1049.5	Luo et al. 2015
J. regia/China	"Yuan Lin" × "Qing Lin"	84/F1	2577 SLAF	16/2457.8	Zhu et al. 2015

interspecific backcross of [*J. hindsii* × *J. regia*] × *J. regia* was investigated. This backcross was from an open-pollinated male-sterile *J. hindsii* × *J. regia* tree ("Paradox Mom") growing in a *J. regia* "Hartley" orchard. Forty-two RFLPs were assigned into 12 LGs, and the estimation of genome size indicated that the walnut genetic map spanned 1660 cM (Fjellstrom and Parfitt 1994a).

Two years later, the same set of progeny was investigated using 66 RAPDs. A new expanded genetic map was created including the previously found RFLPs along with these new RAPD markers. One hundred seven markers were assigned into 15 LGs (Woeste et al. 1996b).

Five years later, an intraspecific cross of *J. regia* was analyzed with both RAPD markers and isozymes. An F_1 progeny of 81 individuals from a *J. regia* "Lara 480" and *J. regia* "Chandler 1036" cross was screened with 120 RAPDs and four isozymes (Malvolti et al. 2001). Only parental maps were created with 11 LGs for the female parent map and 10 for the male parent map.

Next-generation mapping

A 6K SNP iSelect Infinium BeadChip was generated (You et al. 2012) and used, 4 years later, to genotype an F_1 progeny of 425 individuals from an intraspecific cross between "Chandler" and "Idaho" (*J. regia*). A total of 1525 SNPs were assigned into 16 LGs. The LG lengths ranged from 37.7 cM (LG15) to 97.3 cM (LG7), and the length of the genetic map was 1049.5 cM (Luo et al. 2015).

This genetic map was used to construct a walnut physical map with 15,203 exonic BAC-end sequences. This physical map should represent a large part of the genome because its estimated total length, 736.1 Mb, is close to the estimated size of the genome, 606 Mb (Luo et al. 2015). With this mapping work, it has also been possible to find two markers on LG11 which surround the location of the *LB1* gene involved in the lateral bearing phenotype (Dvorak et al. 2015).

Finally, another mapping population, an F_1 progeny of 84 individuals, was created by an intraspecific cross between "Yuan Lin" and "Qing Lin" (*J. regia*). Two thousand five hundred seventy-seven specific-locus amplified fragment (SLAF) markers of three types were used: 2300 SNP only (89.25%), 87 InDel only, and 190 SNP and InDel. They were assigned into 16 LGs, and the integrated genetic map length obtained was 2457.82 cM (Zhu et al. 2015). Moreover, a QTL linked to anthracnose (*Colletotrichum gloeosporioides*) resistance, an important trait in Chinese cultivars, was found on LG14. The QTL interval detected was from 165.51 to 176.33 cM on LG14 with 10 markers and their LOD scores ranging from 3.22 to 4.04.

Genomics and transcriptomics studies

Genomics breakthrough

Genomics is an area within genetics that involves the study of genes and their function, including DNA sequencing, and aims to understand the structure of the genome of an organism. A primary approach to genome analysis is the cloning of exogenous DNA into BAC vectors which can hold genomic DNA fragments up to 350 kb (Choi and Wing 2000). This technique provides a further opportunity for marker development. Using two BAC libraries of 129,024 clones from shoots of "Chandler" grown in vitro and based on 48,218 BAC-end sequences (Table 5) covering 31.2 Mb (5.1% of the genome), 11.5% of the genome was reported to represent coding sequences and a physical map was constructed (Wu et al. 2012).

The same year, 54,912 BAC-end sequences from "Chandler" were generated by Sanger sequencing to identify genome-wide SNPs and to construct the first 6K SNP iSelect Infinium BeadChip (You et al. 2012).

Two years later in China, high-throughput pyrosequencing with Roche technology was used to produce 541,176 reads and 31,362 contigs were assembled covering 15.1 Mb, from which SNPs and InDels were discovered (Liao et al. 2014). In addition, the high-resolution SLAF sequencing (Sun et al. 2013), based on reduced representation libraries (RRLs) and high-throughput sequencing (HTS), was performed in *J. regia* and used to construct a genetic map (Zhu et al. 2015).

Table 5 Summary of walnut genomics and transcriptomics information on the NCBI database (https://www.ncbi.nlm.nih.gov)

Species	Total nucleotide sequences	Nucleotides	EST	GSS	Notes/references
J. regia	223,823	169,18	5253	49,405	40 ESTs unpublished/1–188 ESTs involved in cellular regulation and global metabolism from leaf under irradiance or in darkness/2–5025 ESTs from seed coat tissues/3
J. hindsii × *J. regia*	13,660	101	13,559	0	2715 ESTs from leaves and 10,844 from roots inoculated with *Pratylenchus vulnus* nematodes/4
J. nigra	211	129	78	4	78 ESTs involved in the regulation of heartwood production, related to programmed cell death, from stems/5
J. cathayensis	44	32	12	0	12 ESTs from fresh leaves, buds, female flowers, and male flowers to study genetic diversity/6
All other taxa	601	601	0	0	
Total	238,339	170,043	18,902	49,409	

In another approach, genomic DNA libraries enriched for CA, GA, AAC, and AAG repeats were created in Turkey from "Maras-18" and 624 clones were sequenced (Topçu et al. 2015). The greatest number of alleles and highest levels of polymorphism observed for these *J. regia* were found in the GA-repeats enriched library.

Chloroplast and nuclear genome sequencing

Very important results recently published in walnut genomics are chloroplast genome sequencing (Hu et al. 2016) and nuclear genome sequencing (Martínez-García et al. 2016). The chloroplast genome sequence provides information about phylogeography, genetic diversity, and evolution. The *J. regia* tissue was sequenced using Illumina technology and resulted in the first complete *Juglandaceae* chloroplast genome sequence published (Hu et al. 2016). With a length of 160,367 bp and 36.11% GC content, 137 genes were annotated. Among them, there were 86 genes coding for proteins, three pseudogenes (two *ycf15* and one *infA*), 40 transfer RNA genes (tRNA), and eight ribosomal RNA genes (rRNA). A phylogenetic analysis was performed showing this genome is closely related to others of the *Fagaceae* family.

In 2017, chloroplast genomes of four other Chinese *Juglans* species were investigated (*J. sigillata*, *J. cathayensis*, *J. hopeiensis*, and *J. mandshurica*) by a combination of de novo and reference-guided (Hu et al. 2016—NCBI Accession number KT963008) assembly of whole chloroplast genomes (Hu et al. 2017). Chloroplast genome lengths ranged between 159,714 (*J. hopeiensis*) and 160,367 (*J. regia*) bp with 36.1% GC content and contained 129 genes each, 88 protein-coding genes, 40 tRNA genes, and eight rRNA genes. Seventy-nine genes were chosen for analysis of selective pressure, and five genes were found to be under positive selection. Another recent study indicates some putative mis-annotated and unannotated genes from chloroplast genome sequence of *J. regia* (Chakraborty 2016).

Recently, the phylogenetic position of *J. cinerea*, the unique species of *Trachycaryon* section, was assessed based on chloroplast genomes and nuclear DNA sequences (Dong et al. 2017). Results showed that *J. cinerea* should be included in *Cardiocaryon* section due to its relatedness with *J. mandshurica*, as was also observed with AFLP markers by Fjellstrom and Parfitt (1994b).

The nuclear genome of *J. regia* was sequenced recently and represents the first reference genome sequence for a nut crop. The team of Martínez-García et al. (2016) obtained it from "Chandler" using Illumina sequencing of paired end reads (500 million reads) from short and large fragment libraries. The 667 Mbp were assembled using two methods (SOAPdenovo2 and MaSuRCA) resulting in an N50 scaffold size of 464,955 bp. A total of 221,640 contigs were obtained with 37% GC content. Annotation work led to 32,498 gene models. Among the most abundant molecular functions of annotated genes, were metabolism (25.4%), cellular (22.1%), and membrane components (18.6%) and a set of 10,130 genes had SNPs within their annotated coding sequences. This genome sequence has facilitated construction of a 600K SNP Affymetrix array which is about to become available (David Neale, personal communication, March 2017) and could provide a great impetus for walnut breeding. All of this has been made possible by the establishment of the Walnut Genomics Implementation Group (WGIG) (http://ucanr.edu/sites/wgig/) at Davis, California.

Progress on transcriptomics

Transcriptomics is the study of the complete set of RNAs encoded by the genome at specific times and under specific conditions. ESTs generate millions of short single-pass nucleotide sequence reads for transcriptome exploration (Nagaraj

et al. 2006). Availability of this information in walnut has increased the development of gene-based markers like EST-SSRs which are derived from cDNA libraries. These are more evolutionarily conserved and therefore have higher transferability to related species than non-coding sequences. The first construction of walnut ESTs was accomplished in *J. regia* (Table 5). Five thousand twenty-five ESTs from seed coat tissues covering 16.41 Mb (Muir et al. 2004) have been submitted to NCBI database (https://www.ncbi.nlm.nih.gov). Three years later, an additional 13,559 ESTs were developed (2715 from leaves and 10,844 from roots) from *J. hindsii* × *J. regia* plants infected by *Pratylenchus vulnus* nematodes (Britton et al. 2007).

Next, a *J. nigra* EST library was constructed in order to understand better the determinants of wood quality. Eighty ESTs assumed to be linked to programmed cell death (PCD) were found by analyzing the region of heartwood formation located in the transition zone (TZ) (Huang et al. 2009). A transcript encoding homeobox protein knotted-1-like 3 (KNAT3) was found to be expressed more strongly in the TZ than in other tissues and this transcript, called *JnKNAT3*-like, is 67% similar to *Knox1/Knox2* domains from *A. thaliana* KNAT3.

Three years later, 188 transcript-derived fragments (TDFs) from cDNA-AFLP in *J. regia* became available (Ben Bâaziz et al. 2012). These were obtained as part of a study of light-induced leaf hydraulic conductance (K_{leaf}), which plays a role in plant-regulation of water. A large number of these are linked to protein products involved in global metabolism and cellular regulation. More recently, a transcriptome study of *J. hopeiensis* using Illumina technology identified 39,708 genes (Hu et al. 2015). The same year, 40 brand new ESTs became available in *J. regia* (Zhao et al. 2015).

In 2016, the genetic diversity of Chinese germplasm was assessed using the sequence polymorphisms within the phenylalanine ammonia-lyase (*PAL*) gene (Han et al. 2016). This gene was previously characterized and designated as *JrPAL* (Xu et al. 2012a). Expression of the transcription factor gene *JrCBF* was also studied (Xu et al. 2014). This could have a key role in cold resistance mechanisms because increased expression is observed under cold stress conditions.

Finally, three studies were conducted using Illumina RNA-sequencing methods. The first focused on the transcriptome of the heartwood/sapwood transition zone in *J. nigra* (Chakraborty et al. 2015). A highly abundant transcript in that zone had an amino acid composition linked to a putative extensin, a cell wall protein. The second examined plant-microbe interactions and a large family of NBS-LRR resistance genes in *J. regia* tissues (Chakraborty et al. 2016). The last of these characterized the transcriptome of buds, leaves, female flowers, and male flowers in *J. regia* (Dang et al. 2016).

Metabolomics and proteomics studies

Metabolomics work

Metabolomics is the large-scale study of metabolites, at specific times and under specific conditions, within cells, tissues, or organisms, and provides knowledge about biochemical activity and mechanisms of responses at the molecular level of cells. Over the last few years, some studies have focused on the beneficial effects of walnut consumption on health, directly related to primary metabolites. Unlike several other nuts which contain high level of monounsaturated fatty acids, *J. regia* kernels are rich in polyunsaturated fatty acids (PUFA) such as linoleic (n-3) and linolenic (n-6) acids (Maguire et al. 2004). Several human clinical studies of coronary heart disease have demonstrated that walnut consumption can lead to lower blood LDL cholesterol concentrations (Feldman and Elaine 2002; Banel and Hu 2009). Moreover, a diet with increased PUFA from walnuts is also linked to long-term beneficial effects on type II diabetes (Tapsell et al. 2009). Dietary walnut fingerprinting using HPLC-q-ToF-MS analysis characterized some urinary biomarkers, such as fatty acids metabolites, (Garcia-Aloy et al. 2014) and positive effects of walnut consumption on inflammatory processes, vascular reactivity, glycemic control (Ros 2010), and oxidative stress (Zhao et al. 2014) were described. However, walnuts also can be responsible for food-induced allergies, an emergent public health problem, and techniques to detect and characterize *J. regia* and *J. nigra* allergens have been developed (Costa et al. 2014). Since August 2017, 115 bioactivity screening studies of *J. mandshurica* and *J. regia* have become available on NCBI database (https://www.ncbi.nlm.nih.gov).

Metabolite characterization can also be used for *J. regia* breeding. A study of dynamic metabolite changes during kernel maturation, using gas chromatography-mass spectrometry (GC-MS), investigated changes during kernel ripening (Rao et al. 2016). A total of 252 metabolites were detected and among these, 36.47% were organic acid metabolites, 20% were involved in carbohydrate metabolism, 17.64% in amino acid metabolism, 11.76% in amine metabolism, 7.05% in phosphate metabolism, and 5.88% in lipid metabolism. Higher levels of asparagine, proline, and leucine were found in the early stages of kernel development, involved respectively in nitrogen transport for protein synthesis, prevention of stress-induced damage, and synthesis of derived compounds such as sugars and organic acids. This work showed that a large part of carbohydrate and protein-derived carbon was transferred into other compounds like fatty acids during kernel ripening.

Biosynthesis of nonstructural polyphenols, which could play a role in pathogen resistance, also was studied in *J. regia* (Colaric et al. 2005; Solar et al. 2006; Farooqui et al. 2015). The shikimic acid and aromatic amino acid

biosynthesis pathways generate precursors of numerous secondary metabolites, such as phenolic compounds. In *J. regia*, a major group of polyphenols is the hydrolysable tannins. Polyphenol oxidase (PPO) is responsible for catalyzing oxidation of phenolic compounds including tannins into highly reactive quinones. Originally only a single PPO gene, *JrPPO1* was observed in walnut (Escobar 2013; Araji et al. 2014). Using the genome sequence information, a second gene *JrPPO2* has now been reported and is preferentially expressed in callus tissue (Martínez-García et al. 2016). PPO may be involved in pellicle darkening and silencing the responsible gene could lead to development of walnuts with lighter pellicle color (Butterfield et al. 2015), an important commercial trait.

Proteomics research

Proteomics is the high-throughput analysis of the structure, function, and interaction of proteins produced by the genes of a particular cell, tissue, or organism. In connection with the discovery of the *JrPPO* genes, a tyrosinase was isolated from *J. regia* leaves and identified as a PPO corresponding to the known *JrPPO1* sequence (Zekiri et al. 2014). Tyrosinases can catalyze both ortho-hydroxylation of monophenols to o-diphenols (monophenolase activity) and the subsequent oxidation of o-diphenols to the corresponding o-quinones (diphenolase activity). This *JrTYR* protein shows high monophenolase activity while PPOs of numerous plants lack it. In seeking to elucidate this monophenolase/diphenolase specificity, one hypothesis for the lack of monophenolase activity was structural restriction of active site. However, after the first crystal structure of the *JrTYR* was developed, it appeared that the distinction between the two activities does not depend on the degree of restriction of the active site but, instead, that amino acid residues located at the entrance and in the second shell of the active site play a major role (Bijelic et al. 2015).

Protein markers were developed to study the genetic structure of Pakistan *J. regia* germplasm (Khan et al. 2010). Based on total seed storage proteins analyses, a dendrogram was constructed and accessions were grouped into three clusters. Two accessions were quite different from others and could be useful for future breeding programs.

More recently, a study proposed to fractionate and more widely characterize walnut proteins in order to better understand the alleged health inducing contributions of walnut protein additives for use in food (Mao et al. 2014). Four extractable protein fractions were obtained according to their solubility (glutenin 72.06%, albumin 7.54%, globulin 15.67%, and prolamin 4.73%). These could constitute a good source of essential amino acids for adults. SDS-PAGE analysis showed that molecular weights ranged from 14.4 to 66.2 kDa and two-dimensional gel electrophoresis (2DE) profiles showed that isoelectric points were between 4.8 and 6.8.

Walnut allergen stability during roasting in *J. regia* was investigated by liquid chromatography-mass spectrometry (Downs et al. 2016). Results showed an increase in mature 7S and 11S globulins, known allergens, after roasting for 20 min at 180 °C, probably due to an increase in protein digestibility. Since August 2017, 56,050 protein sequences from *J. regia* are now available on NCBI database (https://www.ncbi.nlm.nih.gov) and will be a useful resource for additional proteomic studies.

Genetic engineering

Since identification of the Ti plasmid in *Agrobacterium tumefaciens* (Schell 1975) and development of the first genetically modified crop plant, an antibiotic-resistant tobacco plant (Fraley et al. 1983), the public acceptance of genetically engineered plants has become a source of debate. Genetic engineering has been used successfully in *J. regia* since methods for somatic embryogenesis from immature cotyledons and plant regeneration were first developed (Tulecke and McGranahan 1985; McGranahan et al. 1988, 1990). Walnut trees expressing a modified *cry1A(c)* gene from *Bacillus thuringiensis* for codling moth (*Cydia pomonella*) resistance were developed in field trials (Leslie et al. 2001). In addition, extensively used "Paradox" rootstock hybrids were modified with *rolABC* genes from *Agrobacterium rhizogenes*, genes of interest in increasing rooting potential (Vahdati et al. 2002).

RNA interference (RNAi) gene silencing also has been used in *J. regia*. The *iaaM* and *ipt* genes of *A. tumefaciens* responsible for crown gall disease were silenced by expressing homologous inducers of post-transcriptional gene silencing (Escobar et al. 2002). Co-transformation with *A. tumefaciens* (*iaaM* and *ipt* genes) and *A. rhizogenes* (*Pv010* gene) was successfully performed in an attempt to combine resistance to both crown gall and *Pratylenchus vulnus* nematode into the same rootstock (gene stacking) (Walawage et al. 2013). *A. tumefaciens* was also used to transfer the *fld* gene from cyanobacteria to *J. regia* in order to increase osmotic stress tolerance (Sheikh Beig Goharrizi et al. 2016).

Improvements in markers for somatic embryo transformed selection have continued in *J. regia*. β-glucuronidase (GUS) was used for most of the early work and has been the most widely employed scorable marker for walnut but a green fluorescent protein (GFP) marker has also been used (Escobar et al. 2000). Recently, a new red fluorescent protein from *Discosoma* sp. (DsRED) was shown to be more stable and reliable (Zhang et al. 2015). DsRED was used to examine gene translocation from transformed rootstock to wild-type scion and this gene was not translocated unlike mRNA was,

suggesting gene signal transport from rootstock to scion in *J. regia* (Liu et al. 2017).

Breeding

In the past, the best walnut genotypes were selected from seedling populations and propagated both for consumption and agricultural traits (McGranahan and Leslie 2012) but walnut breeding programs started in the twentieth century (Fig. 4). The past 50 years have seen the emergence of many workgroups worldwide focused on walnut breeding, genetics, and biotechnology, as well as propagation, nutrition, and production techniques. For access to exhaustive information, see Proceedings of Walnut Symposia: Acta Horticulturae numbers I, 284 (1990, Hungary), II, 311 (1993, Spain), III, 442 (1997, Portugal), IV, 544 (2001, France), V, 705 (2005, Italy), VI, 861 (2010, Australia), and VII, 1050 (2014, China). In general, common goals for walnut breeding are a high yield with ease of shell cracking, kernels of high visual and taste quality, well-adapted flowering and harvest dates, and resistance to major diseases like walnut blight and anthracnose (Cosmulescu and Botu 2012). However, specific characteristics will depend on the area of production and its climatic conditions. The following is a short summary of the main breeding programs that have been, or especially currently are led, by teams in California, France, China, and the Middle East, the most important walnut producing countries.

California breeding program

In the USA, germplasm collections are maintained by the Walnut Improvement Program at the University of California in Davis, CA, and the USDA National Clonal Germplasm Repository in Winters, CA. The UC Walnut Improvement Program objectives include breeding to improve scion cultivars and rootstocks for the California walnut industry, development of better knowledge and methods in order to make breeding more efficient, acquisition, development, and maintenance of diverse germplasm with traits useful for future breeding, and application of new molecular and genomic methods for walnut improvement. Detailed information about the Walnut Improvement Program is available from the Walnut Research Reports Database at the University of California, Fruit and Nut Research Information Center website (http://ucanr.edu/sites/cawalnut/). This breeding program originated from, and remains fundamentally based on, a set of controlled crosses initiated and evaluated by Eugene F. Serr and Harold I. Forde from 1948 to 1979 (Tulecke and McGranahan 1994). Their major goals were to increase yield and kernel quality, to select appropriate leafing and fruiting date, and to increase resistance to diseases. To do

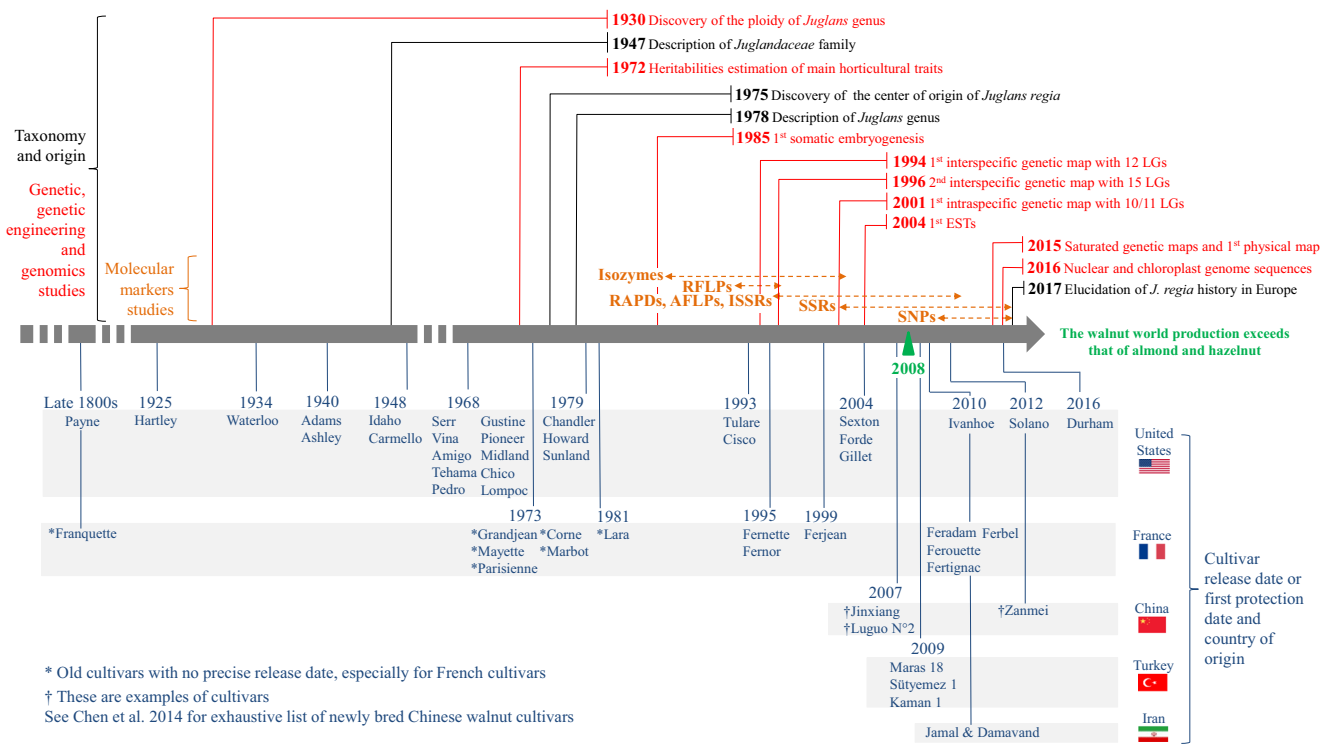

Fig. 4 Timeline of walnut breeding

this, they combined French cultivars for quality and late leafing with California cultivars, such as "Payne," chosen for lateral bearing and precocity. Late leafing helps avoid walnut blight (*X. arboricola* pv. *juglandis*) spread by spring rains. "Payne" is in the background of all current University of California cultivars. After evaluation of about 6000 progeny, ten cultivars were released in 1968. In 1979, an additional three cultivars, "Chandler," "Howard," and "Sunland," were released. The second phase of the breeding program was led by Gale H. McGranahan from 1982 to 2009. Initially directed at blackline resistance, the program objectives were soon expanded to include blight resistance, earlier harvest dates, yield and quality improvement, and rootstock improvement. "Tulare" was released in 1993, and current Californian walnut production is largely derived from "Chandler" (74.2%), "Tulare" (12.6%), and "Howard" (8.1%). Three additional cultivars, "Sexton," "Gillet," and "Forde," were released in 2004. The third phase of the breeding program has been led by Charles A. Leslie since 2009 with the continued goal of obtaining cultivars with early harvest dates, good yield, light kernel color, and resistance to walnut bacterial blight and cherry leafroll virus. Three new cultivars have been released recently: "Ivanhoe" (very early harvesting) in 2010, "Solano" in 2012, and "Durham" in 2016. All three of these cultivars have very light colored kernels and harvest earlier than "Chandler."

The program continues with projects directed at diverse aspects of walnut improvement (Leslie et al. 2015), including (1) Scion cultivar breeding is now emphasizing disease and abiotic stress resistance with 94 repropagated selections under advanced evaluation and more than 3900 seedlings under early stage observation. (2) Backcross breeding is directed at introgression of CLRV resistance into commercial cultivar. A DNA marker linked to CLRV resistance with an error rate of 10% is employed for preliminary screening and preselections are confirmed by direct virus patch testing. Individuals from 4th and 5th generation crosses population are under evaluation. (3) Rootstock breeding for resistance to soil-borne pathogens has emphasized controlled crosses of *J. regia* "Serr" pollen onto selected *J. cathayensis* and *J. microcarpa* female parents and testing for resistance to disease (Kluepfel et al. 2015). (4) An expanded emphasis on applied genomics has included generation and characterization of over 400 "Chandler" × "Idaho" seedlings for marker development, genetic mapping, and development of reference genomes. (5) New technology development includes evaluation of transgenic approaches to disease resistance and continuing improvement of micropropagation methods. (6) Collections of both field and in vitro germplasm resources are maintained for current and future use. Rootstock improvement is concentrated on developing and releasing disease-resistant "Paradox" (hybrids between *J. nigra* and *J. regia*) and improving the clonal propagation procedures needed for commercial production. In recent years, two new rootstocks have been released: RX1 (*J. microcarpa* × *J. regia*) with resistance to *Phytophthora* crown and root rots, and VX211 (*J. hindsii* × *J. regia*) which is very vigorous and shows tolerance to nematodes (McGranahan and Leslie 2012).

French breeding program

In France, germplasm collections are maintained by the Institut National de la Recherche Agronomique (INRA) (in English: National Institute of Agricultural Research) of Bordeaux, more precisely by the Centre for Genetic Resources *Prunus* which also manages *Castanea* and *Juglans* species. The first breeding program was led by E. Germain from INRA, in Unité de Recherche sur les Espèces Fruitières et la Vigne in Bordeaux (in English: Fruit and Vine Research Station) from 1977 to 1995. Twenty-eight crosses were made mainly with French cultivars as female parents ("Franquette," "Grosjean," "Marbot") because they have a late bud break and good fruit quality. Male parents were chosen for lateral bearing including California cultivars ("Pedro," "Chandler") (Germain 1997). Two cultivars were released in 1995: "Fernette" and "Fernor," both lateral bearing cultivars, from a cross between "Franquette" and "Lara." A third new cultivar, "Ferjean," a lateral bearing cultivar with quite small kernels, became available in 1999 from a cross between "Grosvert" and "Lara." In 2010, the last four cultivars from this program were released: "Feradam," "Ferbel," "Ferouette," and "Fertignac." The second French breeding program was led by E. Germain and F. Delort using a large and diverse collection of genetic material developed from 1996 to 2007. The best hybrids from the first program were crossed with genotypes from the Mediterranean area, Iran, Japan, and Central Asia to prevent problems with inbreeding, observed previously among French and California cultivar backcrosses. Although some promising hybrids were characterized and evaluated, the breeding program ended in 2007 due to a new orientation for INRA, following a decision to focus on a smaller number of model species, such as *Prunus* and *Malus*.

About 70% of French walnut production is provided by "Franquette," an ancestral cultivar with late leafing and terminal bearing habit, discovered in the early 1800s. There are two Appelations d'Origine Protégée (AOP) (in English: Protected Designations of Origin) for the two main production areas: AOP Noix du Périgord and AOP Noix de Grenoble. French walnut orchard areas increased by nearly 19% between 2000 and 2010, reaching approximately 20,000 ha. This development makes it the most important French fruit crop other than apples. France exports 80% of its production in-shell, mainly to Europe, thanks in particular to the quality of its product. The main goals of walnut breeding in France are similar to that of California for some traits. The French walnut industry needs new cultivars with lateral bearing, increased yield (~

4 t/ha versus ~2 t/ha for "Franquette"), and larger nut size. New cultivars also need high nut and kernel quality, including light color, thicker shell, and ease of cracking. They must also be well adapted to French climatic conditions, specifically winter hardiness so with late bud break to avoid late spring frosts but also with rapid fruit development. A team of INRA Clermont-Ferrand has been working on winter hardiness and found that the high heritability of autumn and winter hardinesses, and the lack of correlation with growth traits, may provide interesting selection pathways (Guàrdia et al. 2016). However, the development of dedicated models highlighted that early frost damages, although lower than at other times of the leafless period, would negatively influence fruit yield in the following year (Charrier et al. 2017). Last decade, a mechanistic modeling approach has been developed to forecast frost risks under higher climate variability and subsequent stress exposure (Charrier et al. 2015). This approach integrates the relevant physiological processes: dormancy, carbohydrate metabolism, and water status (Charrier and Améglio 2011; Charrier et al. 2013).

A new research project began mid-2017 in cooperation between the INRA Bordeaux-Aquitaine Centre and the Centre Technique Interprofessionnel des Fruits et Légumes (Ctifl) (in English: Fruit and Vegetable Interprofessional Technical Centre), supported financially by the Nouvelle-Aquitaine region. The main objectives are evaluation of the genetic diversity of germplasm resources (about 270 worldwide accessions), identification of the genetic determinants of traits of interest, and establishment of the necessary tools for MAS achievement. In the long term, the choice of genitors for the new breeding program led by the Ctifl will be possible. This project is carried out in several steps including (1) the collection of available data from E. Germain on the germplasm in the form of observation archives about phenology, architecture of the tree, and fruit quality. (2) The phenotyping of the germplasm regarding the three main pests and diseases of *J. regia* in France: walnut blight (*X. arboricola* pv. *juglandis*) and anthracnoses (*Gnomonia leptostyla* and *Colletotrichum acutatum*). (3) The genotyping of the germplasm firstly with SSR markers to eliminate genetically close accessions and secondly with the 600 K SNP Affymetrix array developed by the University of California in Davis to perform genome-wide genotyping on interesting accessions. (4) The completion of a genome-wide association study on traits of interest and the establishment of a germplasm collection.

Other breeding programs

As can be observed in the Proceedings of the Seventh International Walnut Symposium (Tian 2014), a number of other countries also have active walnut breeding programs, notably China and in the Middle East. Chinese breeding goals include early fruiting, high yield with high quality, and tolerance to anthracnose (*C. gloeosporioides*) and other diseases. Twenty-one Chinese provinces or autonomous regions grow walnuts. The major ones are the Xinjiang autonomous region, Yunnan, Shaanxi, Shanxi, Sichuan, Shandong, and Hebei provinces. There is a long cultivation history of walnut in China, and there are many germplasm repositories. Indeed, Chinese botanists have identified and collected more than 800 cultivars, including for example the 216 cultivars, 486 types, and 164 seedling landraces noted by Chen et al. (2014). In 1985, the National Chinese Walnut Germplasm Repository was established at the Shandong Pomology Institute, which currently maintains 415 accessions collected from 24 provinces and from the USA. Each province has developed many local cultivars, but the main ones of national interest include, for instance, "Bofeng," "Bokexiang," "Zha343," "Luguang," "Xiangling," "Hanfeng," "Wen185," the "Jinboxiang" series, the "Zhongling" series, the "Jinlong" series, and the "Luguo" series. Between 2006 and 2012, more than 20 bred cultivars were released, including "Yangza No.1" (tolerant to walnut blight) and "Yangza No.2 hao" (for warm and humid areas) from the Forestry Bureau of Dali in Yunnan in 2006, "Jinxiang" from the Shanxi Academy of Forestry Sciences in 2007 (high yield), "Zanmei" (early bearing), and "Zhenzhuxiang" (small fruits) from the Agricultural University of Hebei in 2012 (Chen et al. 2014). In addition, 17 newly bred cultivars were released by the Shandong Pomology Institute between 2003 and 2012, including "Luguo 2" in 2012 from an intraspecific hybridization and "Luwen 1" in 2009 from an interspecific hybridization (Zhang et al. 2014). Rootstock improvement work also has proceeded in China, including the "Jin RS-1" series released in 2011 by the Shanxi Academy of Forestry Sciences. "Jin RS-2" and "Jin RS-3" have been evaluated for cold, disease, and pest resistance, and seem to be ideal for northern Chinese areas subject to frost (Wang et al. 2014a). Finally, a program in the Xinjiang autonomous region is also assessing the merits of the second bearing habit found in many early ripening dwarf walnuts (Wang et al. 2014b).

In Turkey, walnut germplasm consists mainly of early leafing and terminal bearing cultivars. The production is made mostly in the Mid-eastern Anatolia and Western Black Sea regions. The first Turkish selection study dating to 1971 examined diverse areas of Turkey and 20 seedlings genotypes were selected, including "Yalova 1," "Yalova 3," "Bilecik," and "Şebin" which has become a reference in many production areas of Turkey (Akça 2014). Late leafing and lateral bearing were not yet considered in that work but in 1990, a new selection study was developed in order to select genotypes that would not be damaged by late spring frosts. Selection was conducted for new types combining lateral bearing, late leafing, late flowering, and tolerance to diseases (Akça and Ozongun 2004). At that time, all Turkish cultivars had been selected from native populations (Aslantaş 2006).

The National Walnut Cultivar Breeding Program, initiated in 1998, introduced new cultivars with late leafing and lateral bearing. Then, in 2000, a new breeding program was started to develop cultivars with improved nut quality traits including greater kernel weight and shell thickness (Akça and Polat 2007). In 2008, a variety breeding program directed at developing cultivars with higher yield, late leafing, improved nut quality, and resistance to blight made 13 crosses using Turkish cultivars ("Şebin," "Akça1," etc.) and foreign cultivars ("Franquette" and "Hartley"). From these, 1340 hybrids were obtained and are under evaluation (Akça et al. 2016). From the previous mass selection program, three cultivars were released in 2009: "Maras 18," "Sütyemez 1," and "Kaman 1" (Sütyemez 2016).

In Iran, most walnut trees also originated from seed (Ghasemi et al. 2012). A walnut improvement program started there in 1983 with selection of superior genotypes from native germplasm in Karaj, Shahrood, Mashhad, and Urumieh regions and two major goals: to create new cultivars and to develop efficient vegetative propagation methods (Hassani et al. 2014). Seven superior genotypes were used as parents, and progenies were evaluated along with eight foreign cultivars, including "Serr," "Hartley," etc. As a result, two cultivars were released in 2010: "Jamal" (protandrous) and "Damavand" (protogynous). In addition to these two cultivars, five foreign cultivars ("Chandler," "Pedro," "Hartley" for high yield, "Franquette," and "Ronde de Montignac" for pollination) are recommended for Iranian climatic conditions. Currently, other genotypes are under evaluation for release of new cultivars and development of micropropagation is proceeding (Hassani et al. 2014). To finish, in vitro propagation of walnut plays a very important role in multiplication of cultivars and Iranian teams have been particularly involved in the development of these techniques (Payghamzadeh and Kazemitabar 2011).

Conclusion and future prospects

There is already a long and productive history of walnut genetic improvement since the discovery of heritabilities of main agronomic traits in 1972. Molecular markers permitted the construction of the first genetic maps between 1994 and 2001 and genomics allowed to crack the walnut genome in 2016. The new cultivars, including "Chandler" released in 1979, and the new cropping practices have led to a continuous increase in walnut world production that exceeds since 2008 that of almond and hazelnut. The technological advances permitted to solve a number of questions about taxonomic classification and genetic diversity of worldwide *Juglans* species germplasm.

It is undeniable that "Chandler" genome sequencing now will facilitate and accelerate great advances around the world in walnut genomics, breeding, and identification of genetic determinants of traits of interest. In California, initial genomic selection model building is already planned for 2017 (Neale et al. 2015). The VIII International Walnut Symposium planned for Santiago, Chile in November 2017, will be an opportunity to discover the latest results of teams working on the field. In the near future, we are likely to see the application of CRISPR/Cas9-mediated techniques for walnut improvement, as has already observed in tomato (Pan et al. 2016) and other crops. In France, where the walnut breeding program stopped in 2007, there is now a willingness to initiate a new breeding program that will likely employ MAS, according to Ctifl.

In the context of climate change and global awareness of the ecological impact of chemical inputs, efforts will have to be made in walnut scion and rootstock breeding to release new materials adapted to local environmental conditions and tolerant to current and future pests and diseases, in order to maintain the production and the quality. In addition to genetic improvement, crop management must be continually improved and made more efficient, especially regarding the concepts of water, nutrition, soil, physiology, and defense. In ending words, in light of growing world production and consumption of the crop, rapidly increasing knowledge and use of genetics, new investments in various breeding programs, and widely expanding international research cooperation, there are good times ahead for walnuts.

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