Micropropagation and Identification of Bacterial Endophytes in Interspecific Hybrid Walnuts

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"*Learning is not attained by chance, it must be sought for with ardor and attended to with diligence."*

Abigail Adams

Abstract
Species and hybrids belonging to the genus *Juglans*, e. g., *J. regia* and *J. nigra* x *J. regia* (= *J.* x *intermedia*), deliver highly valuable timber that achieves high prices on international markets. Therefore, the propagation of ortets with outstanding quality for later use in silvicultural programs or plantation forestry is of great interest. Because autovegetative propagation of *Juglans* is difficult to be applied, *in vitro* culture techniques could enable the production of high-quality clones. However, the *Juglans* genus is largely recalcitrant in terms of micropropagation. The reasons for the difficulties include (1) the mature state of the ortets, (2) the general presence of endophytes, probably already in zygotic embryos (3) the slow growth *in vitro* resulting in low propagation rates, and (4) difficulties in rooting of the microcuttings. Thus, the aim of this study was to develop a micropropagation protocol for a large-scale production of a wide range of *J.* x *intermedia* genotypes for later testing and selection of outstanding clones for a multiclonal variety of *J.* x *intermedia*. A second objective was the isolation and characterization of endophytic bacteria from *in-vitro* material which could help to improve micropropagation techniques of *Juglans* clones and the plantlet quality.

Interspecific hybrid walnut genotypes of the F1 offspring originating from open pollination of a single hybridogenic *J. nigra* tree in the nursery of Anton Schott were identified using DNA markers (simple sequence repeats = SSRs). In total, 55 *Juglans* hybrid genotypes, established *in vitro* from embryo axes, were propagated using an optimized tissue culture process with high propagation and rooting rates and also simple acclimatization. The best shoot elongation and production of new axillary shoots was achieved with 4.4 µM BA and 0.2 µM IBA in Rugini (Rugini 1984) and DKW (Driver and Kuniyuki 1984) media, resulting in clone-depending propagation rates from 2.5 to 5.4 per subculture. The optimal gelling agent for shoot cultures was Kobe agar. The highest rooting percentages (50 to 73%) were obtained with a two step rooting protocol (1. induction phase in DKW medium with 12 µM IBA within five days in darkness; 2. root expression under light in a mixture of a gelified ¼-concentrated DKW medium and vermiculite). The survival rates after the acclimatization were 75 to 93%.

To obtain information about the genetic diversity of the *J.* x *intermedia* offspring, population genetic parameters were analyzed for the overall progeny and the contributing pollen and compared to reference data sets. The results of paternity analyses were discussed regarding potential mating preferences of this particular hybridogenic *J. nigra* tree.

Bacterial endophytes in *Juglans in vitro* shoot cultures were analyzed using two different methods: (1) With a culture-dependent approach eight morphologically varying isolates were detected by 16S rRNA sequencing. The findings include *Moraxella* sp., *Brevundimonas* sp., *Acinetobacter* sp., *Pseudomonas* sp. and *Roseomonas* sp. (2) With a culture-independent approach seventeen libraries were established from an amplified 16S rRNA fragment from 51 plant DNA extracts and analyzed using Illumina MiSeq sequencing. More than 85% of the total population of the bacteria was detected in all analyzed samples, some of which are known as plant growth promoting bacteria, such as *Pseudomonas* sp., *Erwinia* sp., *Burkholderia* sp., *Pelomonas* sp., and *Sphingomonas* sp. The genus *Moraxella* sp. was identified in the genotypes with higher propagation rates. This finding is a first step towards future inoculation experiments to prove the proposed beneficial effects during micropropagation of *Juglans* genotypes.

Key words: *Juglans* x *intermedia*, micropropagation, endophytes

Zusammenfassung (Abstract in German language)

Arten und Hybriden der Gattung *Juglans*, z.B. *J. regia* und *J. nigra* x *J. regia* (= *J.* x *intermedia*), liefern wertvolles Holz, das auf dem Weltmarkt hohe Preise erzielt. Daher besteht großes Interesse an der Vermehrung qualitativ hochwertiger Bäume für eine spätere waldbauliche oder plantagenartige Verwendung. Da eine autovegetative Vermehrung von Nussbäumen kaum möglich ist, könnten *In-vitro*-Kulturtechniken die Produktion hochwertiger Klone ermöglichen. Die Gattung *Juglans* erwies sich jedoch in Bezug auf die Mikrovermehrung als schwierig. Die Gründe für solche Schwierigkeiten liegen (1) in der Altersphase der Ausgangsbäume, (2) dem generellen Auftreten von Endophyten, vermutlich bereits in zygotischen Embryonen, (3) den niedrigen *In-vitro*-Vermehrungsraten sowie (4) Schwierigkeiten in der Bewurzelung von Mikrostecklingen. Ziel dieser Studie war deshalb die Entwicklung eines Mikrovermehrungsprotokolls für eine Massenvermehrung eines breiten Spektrums an *J.* x *intermedia*-Genotypen, welches die anschließende Selektion hervorragender Genotypen für eine Hybridnuss-Mehrklonsorte ermöglicht. Ein zweites Ziel war die Isolierung und Charakterisierung endophytischer Bakterien aus *In-vitro*-Material, welche zur Verbesserung der Mikrovermehrung von *Juglans* eingesetzt werden könnten.

Für die Selektion und Mikrovermehrung neuer Nusshybriden (*J.* x *intermedia*) konnten F1-Nachkommen aus freier Abblüte eines hybridisierenden Schwarznussbaumes (*J. nigra*) in der Baumschule Anton Schott genutzt und über DNA-Marker (Simple-Sequence-Repeats = SSRs) identifiziert werden. Insgesamt wurden 55 Nusshybriden nach *In-vitro*-Etablierung aus Embryoachsen über ein optimiertes Gewebekulturverfahren mit hohen Vermehrungs- und Bewurzelungsraten sowie einer einfachen Akklimatisierung mikrovermehrt. Die beste Sprossstreckung und Bildung neuer Achselknospen wurde mit 4.4 µM BA und 0.2 µM IBA in Rugini- (Rugini 1984) und DKW- (Driver and Kuniyuki 1984) Medien erreicht, wobei klonabhängige Vermehrungsaten von 2,5 bis 5,4 erzielt wurden. Das optimale Geliermittel für die Sprosskulturen war Kobe-Agar. Die höchsten Bewurzelungsraten (50 bis 73 %) wurden mit einem Zwei-Schritt-Bewurzelungsprotokoll erzielt (1. fünf Tage Induktionsphase in DKW-Medium mit 12 µM IBA im Dunkeln, 2. Expressionsphase im Licht in einem Gemisch aus festem, ¼-konzentrierten DKW-Medium und Vermiculite). Die Überlebensraten nach der Akklimatisierung lagen bei 75 bis 93 %.

Um Information über die genetische Vielfalt der Nusshybriden zu erhalten, wurden populationsgenetische Daten für die gesamte Nachkommenschaft als auch den Pollenbeitrag erhoben und mit Referenzdatensätzen verglichen. Die Ergebnisse von Vaterschaftsanalysen wurden in Bezug auf die potentielle Bestäubungspräferenz dieser besonderen hybridisierenden Schwarznuss diskutiert. Bakterielle Endophyten in *Juglans*-Sprosskulturen wurden über zwei verschiedene Methoden untersucht: (1) Mit einem kulturabhängigen Ansatz wurden acht morphologisch unterscheidbare Isolate gewonnen und über 16S rRNA-Sequenzierung identifiziert. Zu den Funden zählen *Moraxella* sp., *Brevundimonas* sp., *Acinetobacter* sp., *Pseudomonas* sp. und *Roseomonas* sp. (2) Mit einem kulturunabhängigen Ansatz wurden aus der amplifizierten 16S rRNA von 51 Pflanzen-DNA-Proben siebzehn Sequenzbibliotheken erstellt. Mit Hilfe der Illumina MiSeq-Sequenzierung konnten mehr als 85 % der gesamten Bakterienpopulation erfasst werden, von denen einige als pflanzenwachstumsfördernd bekannt sind, wie *Pseudomonas* sp., *Erwinia* sp., *Burkholderia* sp., *Pelomonas* sp. und *Sphingomonas* sp. In Genotypen mit hoher *In-vitro*-Vermehrungsrate wurde *Moraxella* sp. identifiziert. Dieses Ergebnis ist ein erster Schritt in Richtung künftiger Inokulationsversuche, um mögliche positive Effekte auf die Mikrovermehrung von *Juglans*-Genotypen nachzuweisen.

Schlagworte: *Juglans* x *intermedia*, Mikrovermehrung, Endophyten

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Abbreviations

ANOVA	analysis of variance
ARDRA	amplified ribosomal DNA restriction analysis
BA	benzylaminopurine
CFU	colony forming unit
DGGE	denaturing gradient gel electrophoresis
dNTP	deoxynucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
IBA	indole-3-butyric acid
IFP	Institut für Pflanzenkultur
NGS	next generation sequencing
NW-FVA	Northwest German Forest Research Institute
OTU	operational taxonomic unit
PCR	polymerase chain reaction
PGPB	plant growth promoting bacteria
PGR	plant growth regulator
RAPDs	Random Amplified Polymorphic DNA
rDNA	ribosomal DNA
rRNA	ribosomal RNA
RFLPs	Restriction Fragment Length Polymorphisms
SSRs	simple sequence repeats methods
TGGE	temperature gradient gel electrophoresis
T-RFLP	terminal restriction fragment length polymorphism

1. Introduction

1.1 Classification and distribution of the *Juglans* species

The walnut is classified under the Kingdom *Planta*, Division *Magnoliopsida*, Order *Fagales*, Family *Juglandaceae* and Genus *Juglans*. The *Juglandaceae* family consists of seven genera with approximately 60 species of deciduous trees, including the genus *Juglans, Carya* (pecans and hickories), *Pterocarya* (wingnuts), *Platycarya*, *Engelhardia*, *Alfaroa*, and *Oreomunnea* (Manning 1978). Based on the morphology and phylogeography, twenty-one long-lived deciduous species of the genus *Juglans*, belonging to four distinct sections, have been recorded in the *Juglandaceae* family (Manning 1978). The section *Dioscaryon* Dode contains only one species, *J. regia* L., which is known as the Persian walnut or English walnut and is important for high-quality wood and edible nuts and is distributed from Europe to the Himalayan Mountains (McGranahan and Leslie 1991), while *Rhysocaryon* Dode has 16 species, of which *J. nigra* L. (Eastern black walnut) is one of the well-known North American species. Three species, including *J. ailantifolia* Carr., *J. cathayensis* Dode, *J. mandshurica* Maxim., are found in the section *Cardiocaryon* Dode and are native to East Asia, while *J. cinerea* L., which is naturally distributed in Eastern North America, is found in the section *Trachycaryon* Dode (Manning 1978; Leslie and McGranahan 1992).

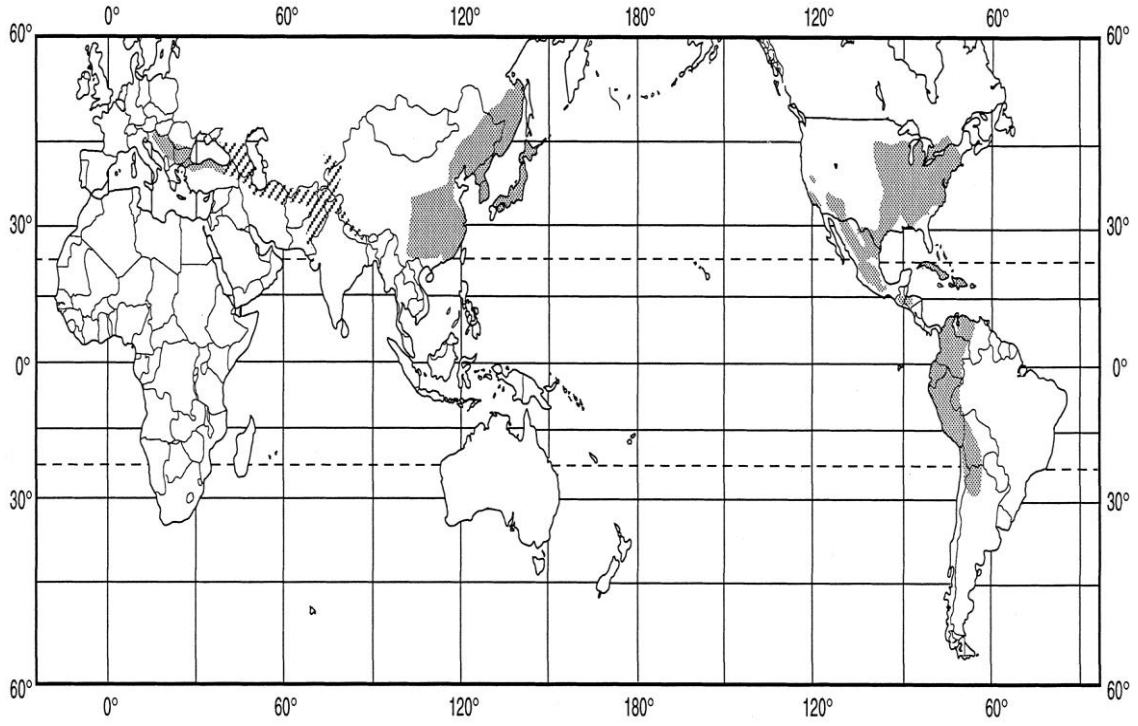

Fig. 1: The distribution of *Juglans* throughout the world (Stanford et al. 2000).

J. regia, *J. nigra* and *J.* x *intermedia (J. nigra* x *J. regia)*

Of the twenty-one *Juglans* species, two species, *J. regia* and *J. nigra*, are most important for nut and wood production and are widely cultivated in the world (McGranahan and Leslie 2009; Pollegioni et al. 2010). Developed for horticulture, *J. regia* (Persian walnut) (Fig. 2), which has been broadly cultivated in many areas of the world not only in North America but from the Iberian Peninsula to the Himalayas and Southcentral China (McGranahan and Leslie 1991), is one of the most valuable tree species for nut and wood production. Persian walnut fruits are a rich source of oils, proteins and vitamins. Dried Persian walnuts can be consumed as snacks or with baked goods as a dessert, and its oils are used in haute cuisine. The most prominent nutrients in walnuts are found to be highly nutritional and beneficial for health, particularly the omega-3 fatty acids (McGranahan and Leslie 2009; Pollegioni et al. 2010). Moreover, their wood can command a high market price for many purposes, including solid wood furniture, veneer, or gunstock production (Leslie and McGranahan 1992).

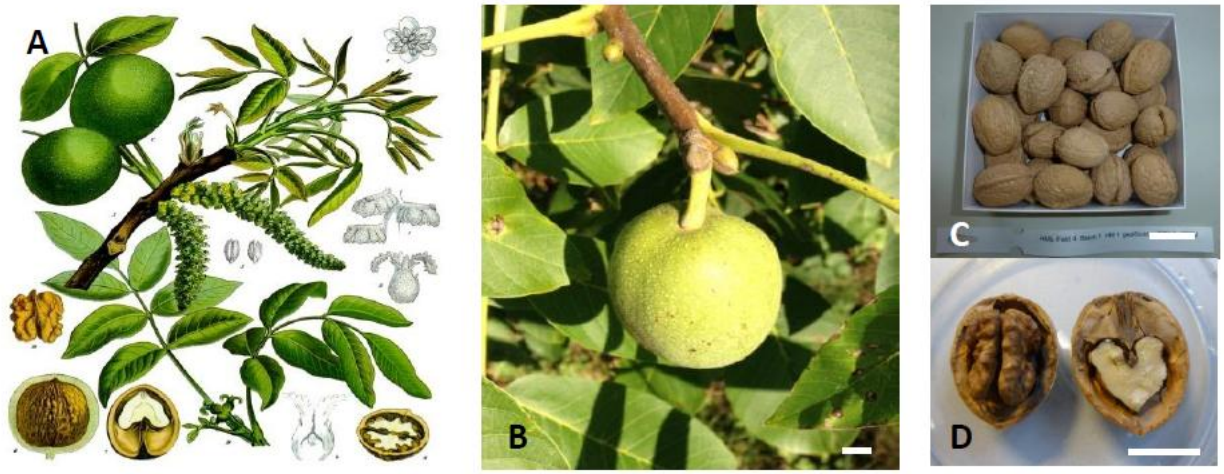

Fig. 2: Persian or English walnut (*Juglans regia* L.) A: Morphological characteristics of *Juglans regia (*http://commons.wikimedia.org/wiki/File:Koeh-081.jpg) B: Mature nut in a hull, Bar= 1 cm; C: Harvested nuts, Bar= 4 cm; D: Nuts with an opened shell, Bar= 2 cm.

Juglans nigra (Black walnut) (Fig. 3), a fast growing species with a high-quality timber and a short rotation time (60 years), is one of the most valuable hardwood species worldwide (Beineke 1983). Because it is hard, durable and straight-grained and is known for its dark luminous beauty, Black walnut wood is often used to make furniture, veneer and specialty products, such as gunstocks, etc. Furthermore, because of its distinct taste, the kernel of the Black walnut is an important food for human consumption, e.g., baked goods and ice cream, and wildlife consumption (Williams 1990).

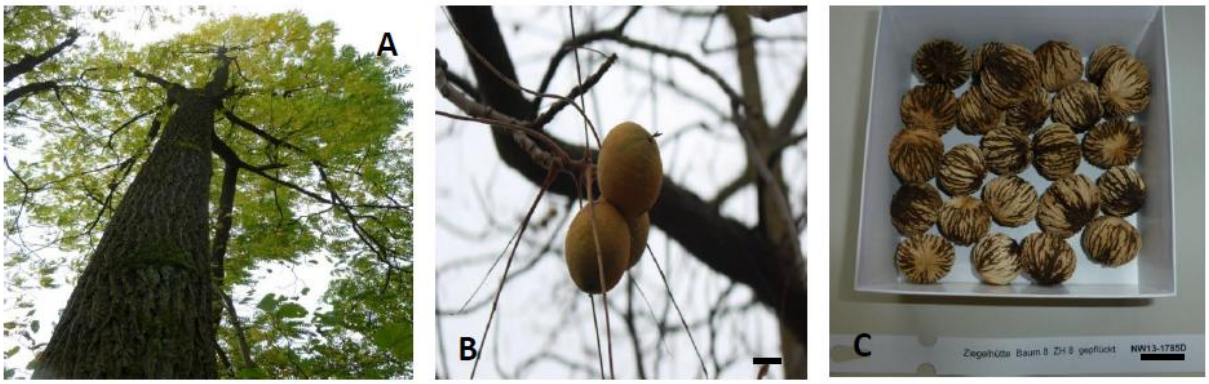

Fig. 3: Morphological characteristics of the Black walnut (*Juglans nigra*) grown in Hann. Münden, Germany. A: Stand of *J. nigra* trees; B: Mature nuts in a hull, Bar= 3 cm; C: Harvested nuts, Bar= 4 cm

Many *Juglans* interspecific hybrids are existing worldwide. For instance, "Leslie Burt" is a cross between *J. nigra* x *J. ailantifolia,* and "buartnuts" are hybrids between *J. cinerea* x *J. ailantifolia* var. *cordiformis*. In Northeast China, the species *J. hopeiensis* Hu is a cross between *J. regia x J. mandshurica.* Moreover, the 'Paradox' rootstock, an interspecific hybrid between *J. regia* and *J. hindsii*, is of the greatest commercial importance. Less vigorous than the Paradox walnut, "Royal" hybrids are hybrids between *J. hindsii* and *J. nigra* (McGranahan and Leslie 2009).

According to McGranahan and Leslie (2009), the Black walnuts in section *Rhysocaryon* will not cross with species belonging to the sections *Trachycaryon* and *Cardiocaryon*. Indeed, the same holds for *J. regia*. The natural and artificial hybrids of *Juglans nigra* (the female parent) x *J. regia* (the male parent), known as *Juglans* × *intermedia* Carr. (Fig. 4), are commercially valuable and function as a crucial rootstock in the industrial walnut production. Compared to the parental species, *Juglans* hybrids show a higher growth potential, distinct disease resistances, good wood quality, strong apical dominance, and lower susceptibility to spring frost damage (Cornu and Jay-Allemand 1989; Fady et al. 2003).

However, walnut hybridization remains rare under natural conditions, and it is difficult to control pollination due to phenological (*J. nigra* pistillate flowers usually mature later than *J. regia* catkins) and genetic incompatibilities (Pollegioni et al. 2012). In addition, adult trees under natural conditions are often too high for phenological observations and controlled pollination. Further, a long time (more than 10 years) is required to observe the overlap of the flowering in the *Juglans* parent trees and select the hybridogenic mother and father trees (Jay-Allemand et al. 1990).

Fig. 4: *J.* x *intermedia* trees in South Germany (Anton Schott). A, B: timber and a *J.* x *intermedia* tree in a 65-year-old stand, respectively; C: Harvested potential hybrids nuts.

1.2 *J.* x *intermedia* identification

Over the last three decades, the hybridogenic parent trees of *Juglans*, which are mother or father trees with a particular aptitude for producing hybrids, have been created by deploying one plus tree (*J. nigra*) as a female parent with several father trees (*J. regia*) to ensure enough pollen for crossing (Pollegioni et al. 2010) and *Juglans* hybrid seeds can be collected from hybridogenic *J. nigra* trees. The interspecific hybrid genotypes can be determined by their morphology, such as leaf or bud shape, in the first year in the nursery. However, the offspring may be either Black walnut if the pollen donor is also a Black walnut (*J. nigra*) or hybrid walnut (*J.* x *intermedia*) if the father is a Persian walnut (see manuscript 2.4).

To date, only limited *Juglans* hybrid plant material is available. Two French interspecific hybrid varieties are NG23 and NG38, which are produced in open-pollinated seed orchards with one female *J. nigra* genotype and two *J. regia* plus tree genotypes as male parents by the French National Institute for Agricultural Research (INRA). These varieties have been commercialized for timber production (Becquey 1990). Another variety, known as "RENI", is produced by a hybridogenic *J. nigra* tree after spontaneous hybridization and has been commercialized by the Anton Schott nursery in South Germany (see 2.2).

The methodologies used for identifying interspecific walnut hybrids involve morphological traits (Jay-Allemand et al. 1990) as well as genetic markers. Enzyme gene markers (e.g. isozymes as a category of biochemical markers) have been applied by Germain et al. (1993) and Hussendörfer (1999). Although isozymes show a codominant gene expression, they have a very low allelic variability and sampling is limited on a restricted number of meristematic tissues (e.g. buds in winter time). Molecular markers, such as Restriction Fragment Length Polymorphisms (RFLPs) and Random Amplified Polymorphic DNA

(RAPDs), have been used by Fjellstrom et al. (1994), Malvolti et al. (1997) and Pollegioni et al. (2010). However, many molecular markers have not usually been used for hybrid identification because they are time-consuming and expensive (RFLPs) or have a low reproducibility (RAPDs), see Pollegioni et al. (2010). Further, RAPDs mostly show dominant inheritance, which is disadvantageous for pedigree and parentage analysis.

Today, a multitude of microsatellites (simple sequence repeats = SSRs) have been characterized for the genus *Juglans* (Woeste et al. 2002, Pollegioni et al. 2009, Zang et al. 2010, Yi et al. 2011, Najafi et al. 2014, Chen et al. 2014, Topçu et al. 2015). The advantages of this type of molecular marker are high variability and codominance allowing to distinguish between homo- and heterozygote individuals and to score the inherited paternal and maternal alleles at any time (Gillet 1999). Thus, SSRs have widely been used, particularly for the genetic characterization of cultivars (for *J. regia* see Dangl et al. 2005), population genetic studies (Victory et al. 2006), or the identification of interspecific hybrids in *Juglans* species (Pollegioni et al. 2010). Also in this study, 55 *Juglans* hybrid genotypes were detected in the offspring of an open pollinated *J. nigra* tree based on six microsatellite loci (WGA027, WGA118, WGA089, WGA331, WGA069, WGA276) (see 2.2 and manuscript 2.4).

1.3 Propagation of *J. regia, J. nigra,* and *J.* x *intermedia*

Because cutting propagation of mature *Juglans* trees is not successful due to the low rooting ability, *Juglans* species are propagated by seeds or grafting onto Black walnut rootstock, which are the predominant methods used in nurseries and breeding programs. However, to date, the results regarding the generative propagation of the *Juglans* species on an Industrial scale demonstrate limited success with a low percentage of seed germination. Moreover, grafting is labor intensive, time-consuming and costly (McGranahan et al. 1988; Payghamzadeh and Kazemitabar 2013). Compared to conventional propagation methods, micropropagation is becoming an important technical achievement for producing new clones of the *Juglans* species by generating a multitude of genetically identical plants in a short time. Over the past ten years, the *in vitro* culture of *Juglans* has proven to be an adequate alternative for classical vegetative propagation. *Juglans* microcuttings have been obtained through shoot-tip multiplication, cultured nodal segments, and somatic embryogenesis (Table 1).

Table 1: Summary of micropropagation studies regarding the *J. regia*, *J. nigra* and *J.* hybrids.

Species	Year of publication	Authors	Explant	Results
J. regia L.	1988	Tulecke	cotyledons	Somatic embryogenesis ; plants
J. regia L.	1989	Revilla et al.	Embryonic axes; nodal segments	Shoots; rooted plants
J. regia L.	1995	Long et al.	Cotyledons	Adventitious regeneration/somatic embryogenesis
J. regia L	1998	Scaltsoyi-annes et al.	Embryonic axes	Micropropagation /germinated embryos, fully developed plant
J. regia L. cv. SU-2	2000	Dumanoğlu	Somatic embryos	Germination of somatic embryos/germinated embryos
J. regia L.	2002	Saadat and Hennerty	Shoot tips	Shoot multiplication
J. regia L.	2004	Vahdati et al.	Somatic embryos	Germinated embryos and development plant
J. regia L. rootstock cv. Perlata	2006	Sánchez-Zamora et al.	Mature fruit	Embryo culture/germinated embryos and development plant
J. regia L	2006	Kaur et al.	Mature fruit	Micropropagation /germinated embryos, fully developed plant
J. regia L.	2008	Vahdati et al.	Microshoots	Rooted plants
J. regia L.	2010	Toosi and Dilmagani	Embryonic axes	Shoot multiplication
J. regia L.	2016	Kepenek et al.	axillary buds	regeneration protocol
J. nigra L.	1986	Neuman et al.	Immature cotyledons	Somatic embryogenesis; callus; roots
J. nigra L.	1986	Heile-Sudholt et al.	Embryonic axes and seedling shoot tips	Shoot multiplication
J. nigra, *J. major* and *J.* hybrids	1988	Cornu	Immature cotyledons	Somatic embryogenesis; inter-specific hybrids
J. nigra × *J. regia*	1989	Cornu and Jay-Allemand	Embryonic axes	Germinant shoots
J. nigra × *J. regia*	1989	Meynier and Arnould	Shoot tips	Rooted plantlets
J. nigra × *J. regia*	1992	Deng and Cornu	Immature cotyledons	Maturation, germination of somatic embryos
Interspecific hybrids	1992	Jay-Allemand et al.	Embryonic axes	Improved rooting of micro shoots; plants
J. regia × *J. nigra*	1993	Barbas et al.	Embryonic axes	Gelling agent effects on shoot growth
J. nigra × *J. regia*	2016	Tuan et al.	Embryonic axes	Shoot multiplication; Gelling agent effects on shoot growth; rooted microcuttings

Juglans belongs to a genus that is more recalcitrant in terms of micropropagation. The reasons for the difficulties include the mature state of the ortets, the general presence of endophytes, which are observed even in early ontogenetic stages and are probably already present in zygotic embryos; the slow growth *in vitro*, resulting in low propagation rates; and difficulties in the rooting of the microcuttings.

Using embryo axes as primary explants, the results of this study indicated that the obvious growth of these *Juglans* hybrid cultures was faster and stronger than that of shoot cultures established with mature explants. Based on literature and own optimization experiments (see manuscripts 2.1, 2.2), the procedure used in this study for *Juglans* micropropagation comprised four main culturing steps (Fig 5):

1. The establishment of *Juglans* shoot cultures from embryo axes. Embryo axes were isolated from the nuts under sterile conditions and placed in a DKW medium (Driver and Kuniyuki 1984) with BA (benzylaminopurine) and IBA (indole-3-butyric acid).
2. Using a DKW (Driver and Kuniyuki 1984) or Rugini (Rugini 1984) medium, the microshoots were sub-cultured every twenty-one days for propagation.
3. Rooting of the *J. regia* microcuttings in media containing IBA, which was applied in the induction phase for five days in the darkness before transferring to the root expression medium (¼ DKW + Vermiculite).
4. After twenty-one days in the expression medium, the *Juglans* microcuttings were acclimatized to the greenhouse conditions.

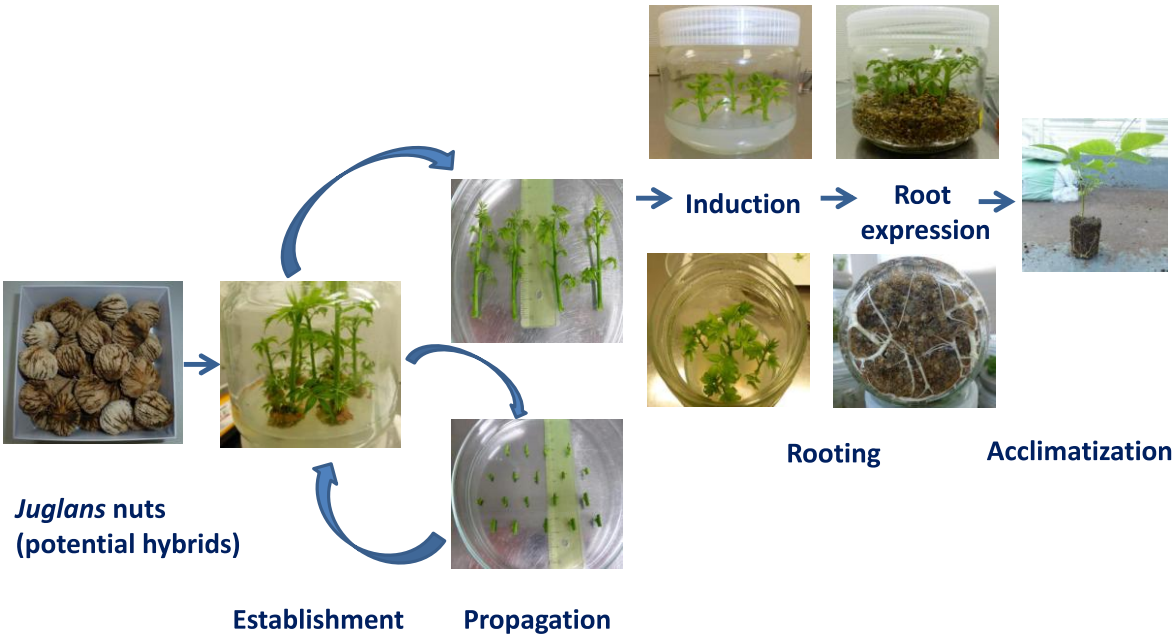

Fig. 5: Micropropagation procedure for *Juglans* species and hybrids.

1.4 Bacterial endophytes in the woody plants

1.4.1 Role of endophytes in the micropropagation of woody plants

According to Petrini (1991), endophytes refers mainly to bacteria or fungi that colonize the internal plant tissue without causing any symptoms of a disease. Previously, studies on bacterial endophytes have focused on agricultural and horticultural plant species such as rice (Reinhold-Hurek and Hurek 1998), sugarcane (Dobereiner et al. 1995), citrus (Araújo et al. 2002) and coffee (Vega et al. 2005). In contrast, endophytic bacteria in woody plants, in general, have not been explored in depth, and almost all studies have concentrated on the effects of bacterial endophytes in the roots on plant growth (Chanway et al. 2000). However, for the past ten years, studies on endophytic bacteria in woody plants were also very encouraging. Because endophytes can have beneficial interaction with their host (Anand et al. 2006), the useful strains for forest practices could increase timber yields to improve environmental quality through enhancement of tree health (Izumi et al. 2008)

Regarding the micropropagation of woody plants, before the 1990s, based on their negative influences, such as browning, early senescence of the plant material (Liu et al. 2005) or their vigorous growth on the surface of the culture media, leading to an overgrowth of the plant (Leifert and Cassells 2001), most bacteria detected in *in vitro* cultures were considered to be "contaminants" (Leifert et al. 1991). However, according to Glick et al. (1998), bacteria can influence plant growth not only directly, such as by providing compounds for the plant or facilitating the uptake of nutrients from the environment, but also indirectly through preventing the deleterious effects of pathogenic organisms. The view on endophytes starts to change, and recently positive effects of the isolated strains that are already known as plant growth promoting bacteria (PGPBs), such as *Pseudomonas* strains or *Paenibacillus* strains were reported, e.g., Ulrich et al. 2008; Quambusch et al. 2014 (Table 2).

Endophytes can have neutral, detrimental or beneficial effects (Hardoim et al. 2015). Some endophytic bacteria can have a beneficial effect on the plant by producing a wide range of phytohormones that play a crucial role in the bacteria-plant interaction, for example, Indole-3-acetic acid (IAA) biosynthesis which is widespread among plant associated bacteria (Spaepen et al. 2007). While, the bacterial strains of genera *Pseudomonas*, *Enterobacter*, *Rhizobium*, *Bradyrhizobium*, *Bacillus*, *Methylobacterium*, *Rhodococcus*, *Acinetobacter*, and *Microbacterium* have been reported (Tsavkelova et al. 2005), another beneficial effect of endophytic bacteria also could be to trigger defense mechanisms

leading to induced systemic resistance by producing insecticidal, nematicidal and antiviral compounds (Kaymak 2010). In in vitro culture, they could be increased nutrient availability, nitrogen fixation or the production of plant hormones.

Table 2: Main genera of endophytic bacteria in the woody plant.

Host plant	Main bacterial genera	Method	Reference
Black locust *Robinia pseudoacacia L.*	*Acidovorax, Dyella, Microbacterium* and *Sphingomonas*	Culture dependent techniques	Boine et al. (2008)
Black locust *Robinia pseudoacacia L.*	*Paenibacillus*	Culture dependent techniques	Ulrich et al. (2008a)
Larix	*Paenibacillus*	Culture dependent techniques	Ulrich et al. (2008)
Picea	*Pseudomonas, Rahnella*	Culture dependent techniques	Ulrich et al. (2008)
Pinus sylvestris	*Methylobacterium*	Culture independent techniques	Pirttilä et al. (2000)
Pinus sylvestris	*Mycobacterium obuense* and *Mycobacterium aichiense*	Culture independent techniques	Laukkanen et al. (2000)
Piper sp., *Taxus baccata* subsp. *wallichiana*, *Withania somnifera*	***Piper nigrum* and *Piper colubrinum:*** *Pseudomonas, Aminobacter, Morococcus, Rhizobacter, Flavobacterium;* ***Taxus baccata subsp. wallichiana:*** *Aminobacter, Pseudomonas, Psychrobacter, Paracoccus;* ***Withania somnifera****: Aminobacter, Pseudomonas.*	Culture dependent techniques	Kulkarni et al. (2007)
Rock plant *Pogonatherum paniceum*	*Mycobacterium cookii.*	Culture independent techniques	Koskimäki et al. (2010)
Poplar *(Populus deltoides x nigra DN34)*	*Methylobacterium*	Culture dependent-independent techniques	Van Aken et al. (2004)
Populus	*Paenibacillus, Agrobacterium rubi, Methylobacterium, Bacillus, Stenotrophomonas*	Culture dependent techniques	Ulrich et al. (2008)
Sour cherry *Prunus cerasus* L. 'Montmorency'	*Pseudomonas syringae* and *Agrobacterium rhizogenes*	Culture dependent techniques	Kamoun et al. (1997)
Prunus avium	*Mycobacterium* sp., *Rhodopseudomonas* sp. and *Microbacterium* sp.	Culture dependent-independent techniques	Quambusch et al. (2014)
Corylus avellana L., *C. avellana* ˈContortaˈ	*Agrobacterium radiobacter* B, *Pseudomonas fluorescens, Xanthomonas* spp., *Enterobacter asburiae, Flavobacterium* spp. and *Alcaligenes* spp.	Culture dependent techniques	Reed et al. (1997)

1.4.2 Detection of bacterial endophytes

By using standard techniques, only a small percentage of bacteria were cultivable (Quambusch 2016). Furthermore, according to Vartoukian et al. (2010) or Schleifer (2004) the "as yet cultivated" bacteria have been estimated below 1% or ranging from 1-10%, respectively. Thus, to identify the bacterial endophytes in micropropagation of *Juglans* species, it was necessary to consider both the cultivable and non-cultivable fractions of bacterial endophytes.

The culture-dependent approach for identifying cultivable bacteria began with placing the plant material (leaf or stem from the tissue cultures) on plates with the culture media. After five weeks of monitoring, the obtained bacteria were purified by re-streaking the bacterial colonies into a fresh medium to obtain single colonies. DNA was extracted from the bacteria, a 16S rRNA fragment was amplified by PCR, and the obtained sequences were compared with sequences available in the NCBI GenBank database (Fig 6).

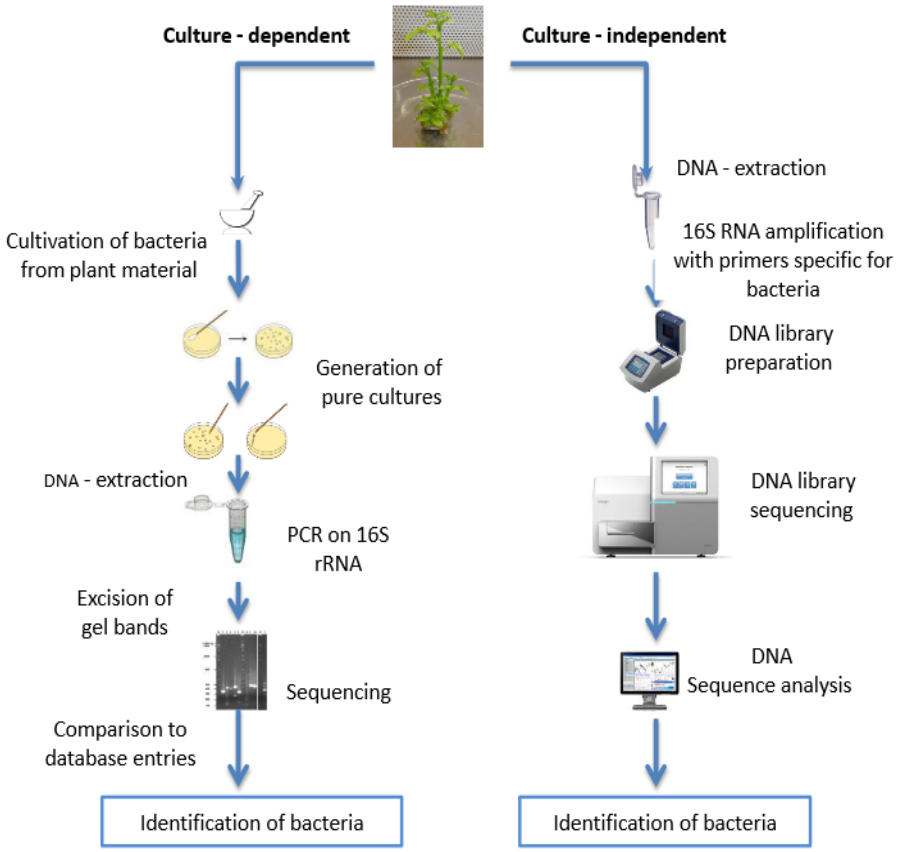

Fig. 6: Procedures used in this thesis to identify the bacterial endophytes in *Juglans in vitro* cultures by culture-dependent and culture-independent methods [The graphic modified after Quambusch (2016)].

The culture-independent approach involving Illumina MiSeq 16S rRNA amplicon sequencing, which can detect millions of small sequence reads simultaneously, was applied in this study. DNA was extracted directly from the plant material, and the subsequent workflow included the following five steps: 16S rRNA amplification with primers specific for bacteria, sequencing library preparation, cluster generation, sequencing and data analysis (see details in section 2.2 and Fig. 6).

1.5 Thesis objectives

The objectives of this study were:

1. Application of microsatellite (SSR) marker techniques for a precise species differentiation to detect hybrids in early ontogenetic stages (embryo tissue) for micropropagation and to calculate the genetic diversity of hybrid progeny to provide a broad genetic base for further testing and selection of elite genotypes on different ecological sites
2. Development of an improved protocol for the micropropagation of *Juglans* shoot cultures for large-scale production:
   a. Evaluate different culture media varying in nutrient composition and cytokinin concentration for the multiplication of *Juglans (J. regia* and *J.* x *intermedia)* shoot cultures
   b. Evaluate solid and liquid medium (*J. regia*) and three gelling agents (*J. x intermedia)* for axillary shoot multiplication
   c. Identify the optimal IBA concentration for the root induction phase and determine the optimal of different root expression media for the *Juglans* microcuttings
3. Identify bacterial endophytes and a possible correlation between the bacterial populations with the propagation success *in vitro* in the shoot cultures of sixteen different *Juglans* genotypes
   a. Establishment of a protocol for the identification of bacterial isolates by using culture-dependent methods.
   b. Identification of the total bacterial community structure in the shoot cultures of *Juglans* by using culture independent (Illumina MiSeq sequencing) methods.
   c. Correlate the differences between the bacterial populations of the different genotypes with the propagation success *in vitro*.

2. Publications and Manuscripts

Manuscript 2.1

2.1 Factors affecting shoot multiplication and rooting of Walnut (*Juglans regia*) *in vitro*

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Type of authorship:	First author
Type of article:	Proceeding article
Contribution to the article:	Planned and performed all experiments Analyzed the data Prepared all figures Wrote the paper
Contribution of other authors:	*Andreas Meier-Dinkel*, *Aki. M. Höltken*, *Irene Wenzlitschke*, and *Traud Winkelmann* contributed in design of experiments, data analysis and writing the paper
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Factors affecting shoot multiplication and rooting of walnut (*Juglans regia* L.) in vitro

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Abstract

***Juglans regia* is one of the most important nut crops rich in oil, protein and vitamins, and its high value timber is used for furniture, veneer and gunstock production. However, the propagation of *J. regia* is strongly dependent on the genotype. Slow in vitro growth results in low propagation rates and difficulties are encountered using conventional rooting methods. Embryo axes dissected from mature nuts were used as primary explants for establishing shoot cultures. The growth of walnut shoot cultures was compared on media differing in nutrient formulation (Rugini olive medium and DKW medium), concentrations of 6-benzyladenine (BA) (2.2-8.8 µM), solid and liquid medium and indole-3-butyric acid (IBA) concentration (4-20 µM). During the 21 days culture passage the Rugini medium gave significantly higher fresh mass and numbers of axillary shoots than DKW medium. The best BA concentration for *J. regia* explants was 2.2 µM BA. The results also indicated that solid medium (7 g L^{-1} agar) was better than liquid medium for shoot proliferation. The optimal IBA concentration in the induction medium for rooting of microcuttings was 12 µM IBA applied for 5 days in darkness before transfer to root expression media. There was no significant difference among two root expression media (¼ DKW + Vermiculite and ¼ MS + Vermiculite) in rooting of microcuttings after 21 days. The acclimatization of rooted microcuttings was difficult because the tendency to dessicate of plantlets and because of the occurrence of diseases due to high humidity in the greenhouse. Nevertheless, 80% of the *J. regia* plantlets were successfully established in the greenhouse.**

Keywords: benzylaminopurine, DKW medium, indole-3-butyric acid, root induction, root expression, Rugini olive medium

INTRODUCTION

Walnut (*Juglans regia* L.) is one of the world's most valuable tree species for nut and wood production. Nuts are a rich source of oil, protein, vitamins and their wood is used for furniture, veneer, and gunstock production with high commercial value. However, the main problems with in vitro propagation are long propagation cycles and low rooting percentages. (Cornu and Jay-Allemand, 1989; Leslie and McGranahan, 1992; Toosi et al., 2010) .

The aims of this study were to evaluate different culture media varying in nutrient composition and cytokinin concentration, to compare between solid and liquid medium, to identify the best IBA concentration in the root induction phase and to find out the best basal medium in the root expression phase for *J. regia* microcuttings.

MATERIAL AND METHODS

Nuts of Persian walnut (*J. regia* L.) were harvested in Hann. Münden, Germany. The epicarp was removed and the nuts were disinfected for 2 min in 70% ethanol, then for 20 min in 5% NaOCl with several drops of Tween and finally washed in sterile distilled water. Embryo axes were isolated from the nuts under sterile conditions and quickly dipped in

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70% ethanol followed by immersion in 0.5% NaOCl with several drops of Tween 20 for 5 min. The embryo axes were rinsed two times in sterile water and placed on DKW medium with 4.4 µM 6-benzyladenine (BA) and 0.2 µM indole-3-butyric acid (IBA) (Payghamzadeh and Kazemitabar, 2010) (Figure 1a and b).

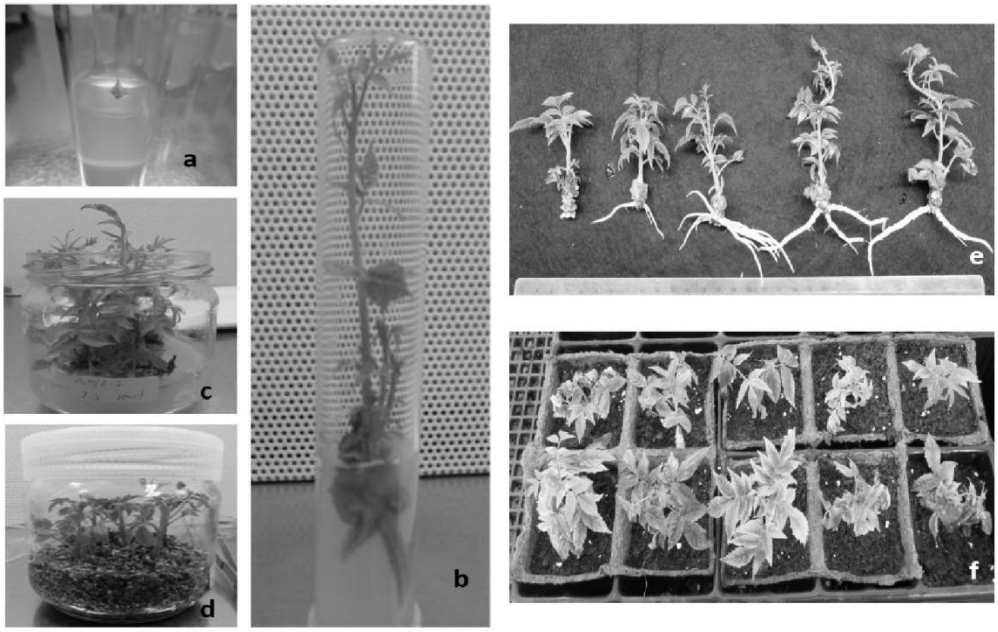

Figure 1. Micropropagation of *Juglans regia*. (a) Embryo axes; (b) Germinating embryo axes; (c) Propagation phase; (d) Rooting with vermiculite medium; (e) In vitro rooted shoots of *J. regia,* (f) Plantlets of *J. regia* established in the greenhouse.

All media were sterilized by autoclaving at 121°C and 1.0 kg cm^{-2} pressure for 20 min. The pH was adjusted to 5.7 prior to autoclaving. Cultures were maintained in a growth chamber under a 16-h photoperiod and temperatures of 27±1°C under cool white fluorescent lamps at 75 µmol m^{-2} s^{-1}.

Shoot cultures of one established genotype were used for the evaluation of two basal media (pre-prepared Duchefa), DKW medium (Driver and Kuniyuki, 1984) and Rugini olive medium (Rugini, 1984) (Table 1).

The cytokinin BA was supplied in three concentrations of 2.2, 4.4 and 8.8 µM. Glucose was used at 30 g L^{-1} and Kobe agar (Serva Co.) at 7 g L^{-1}. The shoots were cultured in 250 mL glass jars containing 80 mL of solidified medium. At the end of the experiment (after 21 days) the following parameters were measured: callus fresh mass, shoot fresh mass and main shoot length and number of axillary buds. Each experiment consisted of a completely randomized design with five replicates, each replicate containing five explants (Figure 1c).

In the following experiment, the best culture medium from the first experiment was used (Rugini medium with 2.2 µM BA). In the multiplication phase, shoots of *J. regia* were cultured in solid (7 g L^{-1} Kobe agar) and liquid medium (without agar) by the static conventional system and the explants were inserted vertically to the depth of a net above the liquid medium. The parameters evaluated were the same as described above.

According to the technique described by Jay-Allemand and Cornu (1986) for rooting of *J. regia*, two phases were assessed: (1) Induction phase: microcuttings were placed on root induction medium in the dark at 27°C for 5 days before transferring to root expression medium. The induction medium was supplemented with 4 µM, 12 µM and 20 µM IBA, respectively, 30 g L^{-1} glucose and 7 g L^{-1} Kobe agar (2) Expression phase: after the comparison between the three IBA concentrations, two root expression media (¼ DKW + Vermiculite and ¼ Murashige and Skoog (1962, MS) + Vermiculite) were compared. The parameters evaluated were main root length and root number per rooted shoot.

Table 1. Composition of the tissue culture media used for walnut shoot multiplication. Source: Driver and Kuniyuki (1984) and Rugini (1984).

Nutrient	Culture media	
	DKW	Rugini olive medium
Macronutrients	(mg L^{-1})	(mg L^{-1})
NH_4NO_3	1416.00	412.00
$CaCl_2$	112.50	332.16
$Ca(NO_3)_2 \times 2H_2O$	1664.64	-
$Ca(NO_3)_2$	-	416.92
KH_2PO_4	265.00	340.00
K_2SO_4	1559.00	-
KNO_3	-	1100.00
$MgSO_4$	361.49	732.60
KCl	-	500.00
Micronutrients		
H_3BO_3	4.80	12.40
$CuSO_4 \times 5H_2O$	0.25	0.25
$MnSO_4 \times H_2O$	33.80	16.90
$Na_2MoO_4 \times 2H_2O$	0.39	0.25
$ZnSO_4 \times 7H_2O$	17.00	14.30
KI	-	0.83
$CoCl_2 \times 6H_2O$	-	0.025
FeNa EDTA	44.63	36.70
Vitamins		
Nicotinic acid	1.00	5.00
Thiamine HCl	2.00	0.50
Pyridoxine	-	0.50
Myo-inositol	100.00	100.00
Biotin	-	0.05
Folic acid	-	0.50
Glycine	2.00	2.00

For the acclimatization process, plantlets of *J. regia* from in vitro rooting (from both ¼ DKW + Vermiculite and ¼ MS + Vermiculite expression medium) were directly inserted in a mix of peat: perlite (1:1) with an intermittent fog system. The humidity in the greenhouse was 100% from the 1st to the 14th day, followed by 90% from the 15th to the 21st day. The temperature was 24/21°C (day/night). The survival of the plantlets was assessed after 21 days.

The results were analyzed according to analysis of variance, and separation of means was tested using Tukey's HSD (Honestly Significant Difference) test, at the level $P<0.05$ by the Statistica 12 (StatSoft) program.

RESULTS AND DISCUSSION

Our data indicate that both DKW and Rugini medium, supplemented with the lowest BA concentration of 2.2 µM resulted in a significantly higher main shoot length and a higher number of axillary buds (4.3 and 4.5 cm, 7.9 and 11.7 buds, respectively) after 21 days of culture (Table 2). Based on mean values, the most appropriate culture medium and cytokinin concentration for the in vitro multiplication appeared to be Rugini medium with 2.2 µM BA (Figure 2). Thus, differential nutrient content in the culture media (Rugini medium contains more Ca^{2+} and K^+ but less NH_4^+ and SO_4^{2-} than DKW medium) may have a significant effect on shoot development. This finding is consistent also with other investigations (see e.g., Šedivá et al., 2013 on horse chestnut, *Aesculus hippocastanum*).

Table 2. Growth of *J. regia* explants after 21 days of culture in different nutrient media and concentrations of BA.

Nutrient medium (µM)	Callus fresh mass (g)	Shoot fresh mass (g)	Main shoot length (cm)	No. axillary buds
DKW				
2.2 BA+0.2 IBA	1.19±0.79a[1]	0.45±0.21a	4.34±1.00a	7.88±2.67a
4.4 BA+0.2 IBA	1.96±1.01b	0.59±0.56ab	3.20±1.84b	7.76±3.38a
8.8 BA+0.2 IBA	1.97±1.26b	0.27±0.28a	1.20±0.99c	3.92±3.04b
Rugini				
2.2 BA+0.2 IBA	1.38±0.62a	0.52±0.19a	4.49±0.99a	11.72±3.5a
4.4 BA+0.2 IBA	2.18±0.78b	0.66±0.30ab	3.70±1.53a	9.44±2.41b
8.8 BA+0.2 IBA	1.20±0.52a	0.44±0.24a	1.54±1.23b	8.00±3.51b

[1]Values followed by the same letter within the same column (and nutrient medium) are not significantly different according to Tukey's HSD test at $p<0.05$. Data are expressed as mean ± SD.

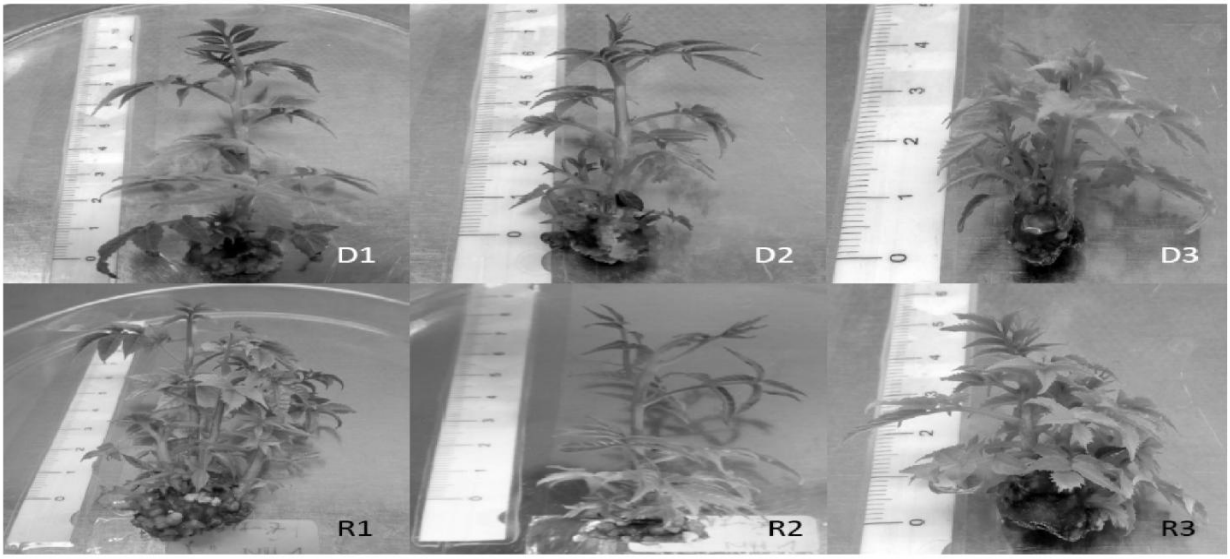

Figure 2. *J. regia* explants after 21 days of culture in different nutrient media and concentrations of BA. D1-3: DKW medium with 2.2 µM BA + 0.2 µM IBA (D1), 4.4 µM BA+ 0.2 µM IBA (D2) and 8.8 µM BA + 0.2 µM IBA (D3). R1-3: Rugini medium with 2.2 µM BA + 0.2 µM IBA (R1), 4.4 µM BA + 0.2 µM IBA (R2) and 8.8 µM BA + 0.2 µM IBA (R3).

Furthermore, significant differences were found for growth parameters of the *J. regia* explants after 21 days when using solid and liquid Rugini medium containing 2.2 µM BA + 0.2 µM IBA (Table 3). More axillary buds and longer shoots were obtained in the solid medium with 7 g L^{-1} agar than in liquid medium (9.2 against 5.4 buds and 3.3 against 2.1 cm shoot length, respectively). However, we could observe that the shoots in liquid medium appeared fresher and greener than in solid medium and hyperhydricity was observed on shoots in liquid medium. This is in accordance with observations on *Dianthus caryophyllus* (carnation) and *Cynara cardunculus* var. *scolymus* (artichoke): hyperhydricity may be a result of too high humidity in the medium or of growth on a liquid medium (Debergh et al., 1981).

Table 3. Growth of *J. regia* explants after 21 days of culture in liquid and solid Rugini medium with 2.2 µM BA and 0.2 µM IBA.

Gelling agents	Callus mass fresh (g)	Shoot fresh mass (g)	Main shoot length (cm)	No. axillary buds
7 g L^{-1} agar	0.99±0.46a[1]	0.46±0.19a	3.27±0.19a	9.24±3.14a
No agar	0.47±0.27b	0.26±0.16b	2.12±0.39b	5.40±2.78b

[1]Values followed by the same letter within the same column are not significantly different according to Tukey's HSD test at $p<0.05$. Data are expressed as mean ± SD.

Following the two-phase of rooting procedure described above, the rooting rate on ¼ DKW + Vermiculite expression medium was higher (56%) than on ¼ MS + Vermiculite expression medium (47%) (Figure 3) after 21 days and there was no significant difference in main root length in ¼ DKW + Vermiculite and ¼ MS + Vermiculite expression medium (Table 4). However, in ¼ DKW+ Vermiculite following induction with 12 µM IBA the significantly highest root number per rooted shoot of 2.1 was obtained, whereas the differences observed on ¼ MS + Vermiculite expression medium were not significant for all three IBA concentrations applied during induction (Table 4; Figure 1d, e).

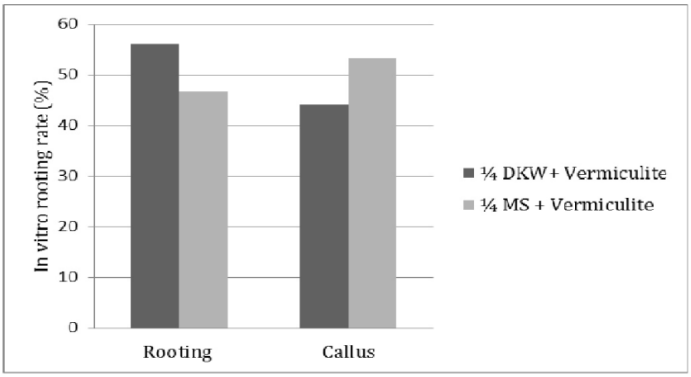

Figure 3. In vitro rooting rate of *J. regia* microcuttings after 21 days in expression medium.

Table 4. Effect of IBA concentration in the induction medium and of nutrient composition in the expression medium on rooting of *J. regia* microcuttings after 21 days.

Rooting medium	Main root length (cm)	Root number per rooted shoot
¼ DKW + Vermiculite (expression)		
4 µM IBA (induction)	0.80±1.22a[1]	0.52±0.77b
12 µM IBA (induction)	1.33±1.17a	2.08±1.80a
20 µM IBA (induction)	0.41±0.85a	0.56±1.08b
¼ MS + Vermiculite (expression)		
4 µM IBA (induction)	0.80±1.13a	0.68±0.98a
12 µM IBA (induction)	1.52±1.54a	1.48±1.58a
20 µM IBA (induction)	0.91±1.47a	0.52±1.08a

[1]Values followed by the same letter within the same column (/nutrient medium) are not significantly different according to Tukey's HSD test at $p<0.05$. Data are expressed as mean ± SD.

During acclimatization in the greenhouse more than 80% of the plantlets from in vitro rooting (for both DKW and MS root expression media) continued to grow vigorously (Figure 1f), whereas the rest of the plantlets collapsed after transplanting to the greenhouse. Furthermore, 71.4% of the plantlets with callus continued to grow while 28.6% of the plantlets with callus died immediately or temporarily survived during acclimatization (21 days) without producing any further growth (Figure 4).

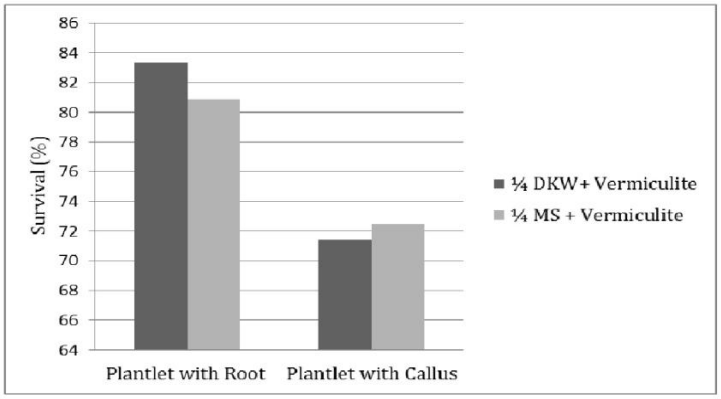

Figure 4. Plantlet survival rate of *J. regia* microcuttings after 21 days in greenhouse.

CONCLUSIONS

The use of Rugini medium supplemented with 2.2 µM BA, 0.2 µM IBA and 7 g L^{-1} Kobe agar is suggested for in vitro shoot proliferation of *J. regia* starting from embryo axes. For in vitro rooting, the best IBA concentration in the induction medium was 12 µM IBA for 5 days in darkness before transferring the *J. regia* microcuttings to root expression medium. There was no significant difference among two root expression media (¼ DKW and ¼ MS) after 21 days.

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Manuscript 2.2

Paving the way for large-scale micropropagation of *Juglans* × *intermedia* using genetically identified hybrid seed

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Type of authorship:	First author
Type of article:	Research article
Contribution to the article:	Planned and performed all experiments Analyzed the data Prepared all figures Wrote the paper
Contribution of other authors:	*Andreas Meier-Dinkel*, *Aki. M. Höltken*, *Irene Wenzlitschke*, and *Traud Winkelmann* contributed in design of experiments, data analysis and writing the paper
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ORIGINAL ARTICLE

Paving the way for large-scale micropropagation of *Juglans* × *intermedia* using genetically identified hybrid seed

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Abstract This study provides a basic tool for the production of a multiclonal variety of *Juglans nigra* × *J. regia* hybrids (*J.* × *intermedia*), including optimized in vitro propagation methods and DNA-based techniques for the identification of hybrid seeds. Following identification of hybrid seed material using DNA markers embryo axes dissected from mature nuts were used as primary explants for establishing shoot cultures. The growth of shoot cultures of four hybrid genotypes was compared on two different basal media (Rugini and DKW) with three concentrations (2.2; 4.4; and 8.8 µM) of 6-benzylaminopurine (BA), and three gelling agents (Oxoid agar, Kobe agar, Gelrite). Three indole-3-butyric acid (IBA) concentrations (4, 12, and 20 µM) were compared for root induction, as well as three media for root expression. In general, the best combination of shoot elongation and production of new axillary shoots was achieved with 4.4 µM BA and 0.2 µM IBA in Rugini and in DKW medium. However, shoot elongation in most genotypes was favored when 2.2 µM BA and 0.2 µM IBA was used, in both, DKW and Rugini medium. The optimal gelling agent for *Juglans* hybrid shoot cultures was Kobe agar in Rugini medium. Highest rooting percentages were obtained on DKW medium with 12 µM IBA with 5 days in darkness followed by root expression under light on a mixture of gelified ¼ DKW medium and vermiculite. During acclimatization, more than 75 % of the plantlets continued to grow vigorously.

Keywords *Juglans* hybrids · DNA fingerprinting · In vitro propagation · Shoot proliferation · Rugini medium · Rooting

Introduction

Juglans regia L. (Persian walnut), native from Europe to the Himalayan Mountains, is one of the most valuable tree species in terms of wood and nut production and it is widely cultivated in many areas of the world. Species timber is used for furniture, veneer and gunstock production with high commercial value (Leslie and McGranahan 1992). However, its low competitive ability, compared to tree species such as beech, maple or ash and its susceptibility to spring frost and forest pathogens, e.g. honey fungus (*Armillaria mellea*), makes the silvicultural treatment difficult and expensive. The Persian walnut (*J. regia*) has been commercialized by several companies including L&J BioTech (France) in some small markets and made profits due to lack of competitive structure. An alternative is *J. nigra* L. (black walnut), as well a very valuable hardwood timber species native to North America. Though, more resistant and tolerant to biotic and abiotic stressors and fast growing with a short rotation time of about 60 years, this tree species requires best soil conditions, particularly alluvial sites, and it has a mostly uniform dark wood color (Beineke 1983).

However, their hybrids (*J. nigra* × *J. regia* = *J.* × *intermedia*) appeared to be a very interesting alternative for

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forestry due to outstanding characteristics combining most of the positive features of both parental species: For example, distinct vigorous growth and thus sufficient competitive ability, straightness of boles, more 'regia' typic coloration of the timber, site tolerance and resistance towards biotic and abiotic hazards (Fady et al. 2003; Jay-Allemand et al. 1989; Mettendorf 2008). Unfavorably, hybridization between these two species is rare under natural conditions due to genetic and phenological incompatibilities (e.g. differing flowering periods). Hence, only limited plant material was available over the last decades: the offspring of a *J. nigra* tree after spontaneous hybridization in South Germany (commercialized as RENI, Anton Schott nursery) and two French hybrid varieties (commercialized as NG23 and NG38, French National Institute for Agricultural Research (INRA) selection). In order to produce a multiclonal variety of tested genotypes for further field experiments and later utilization in forestry, micropropagation seems to display the only appropriate tool for an efficient large scale propagation system. Up to now, there is no evidence that vegetatively propagated *Juglans* hybrids (*J. nigra* × *J. regia*) have been traded. This technique, combined with a DNA-based hybrid identification method, offers the potential of developing an efficient, time-saving multiplication procedure of genetically variable hybrid material from early ontogenetic stages (mature embryos). Further, biochemical and molecular markers are available to distinguish between *J. regia*, *J. nigra* and interspecific hybrids using embryonic tissue (Emilia et al. 1995; Fornari et al. 1999; Hussendorfer 1999; Pollegioni et al. 2009).

Preliminary results concerning micropropagation of *Juglans* hybrids are promising such as germination of embryonic axes (Cornu and Jay-Allemand 1989), production of rooted plantlets (Meynier and Arnould 1989), maturation and germination of somatic embryos (Deng and Cornu 1992), rooting of microshoots (Jay-Allemand et al. 1992) and testing the effects of gelling agents on shoot growth (Barbas et al. 1993). Beside classical micropropagation via axillary shoot formation somatic embryogenesis could be an alternative approach of propagation. However, Vahdati et al. (2008) working with *J. regia* could not solve problems of somatic embryo germination. Recently, detailed comparisons of somatic and zygotic embryos of *J. regia* were carried out for total protein, proline content, total protein spectra (SDS-PAGE), activities of some antioxidant enzymes including peroxidase (POX), catalase (CAT), superoxide dismutase (SOD) and polyphenol oxidase (PPO) in order to overcome still existing limitations in propagation via somatic embryogenesis (Jariteh et al. 2015). Deficiencies during maturation of somatic embryos were detected and application of abscisic acid and proline were suggested to be tested for optimizing somatic embryo maturation.

Several issues of propagating *Juglans* hybrids in vitro are still unsolved such as irregular or often low rooting rates (Rodriguez et al. 1989). However, the experiments reported here were undertaken to select new genotypes of interspecific hybrid walnuts, to develop a micropropagation protocol optimized for *Juglans* hybrids with high propagation and rooting rates and simple acclimatization which is suitable for large-scale production for forest plantations.

Overall, in order to develop an efficient mass propagation system for *J.* × *intermedia*, this study deals with the following topics:

1. SSR markers were used to identify and characterize interspecific *Juglans* × *intermedia* embryos from an open pollinated tree;
2. Different steps of the micropropagation protocol were optimized, e.g. by evaluating two basic tissue culture media, different BA concentrations and gelling agents for axillary shoot multiplication, as well as IBA concentrations during root induction, and different expression media.

Materials and methods

Plant material

Embryo preparation

Shoot cultures were established from embryo axes of 38 mature nuts collected from a single black walnut tree from a nursery in South Germany (Anton Schott) which in previous years had given rise to hybrid nuts. In a first step, the epicarp was removed in order to collect the nuts. These nuts were disinfected in 70 % ethanol for 2 min, followed by 5 % NaOCl with several drops of Tween 20 for 20 min and then washed in sterile distilled water. After isolating embryo axes from the nuts under sterile conditions, a small amount of embryo tissue was sampled for species identification by DNA analysis before the material was quickly dipped in 70 % ethanol, followed by immersion in 0.5 % NaOCl with several drops of Tween 20 for 2 min. Finally, the embryo axes were rinsed twice in sterile water and placed on DKW medium (Driver and Kuniyuki 1984) with 4.4 µM 6-benzylaminopurine (BA) and 0.2 µM indole-3-butyric acid (IBA) (Payghamzadeh and Kazemitabar 2010).

Reference material

In order to identify the potential hybrid status of the prepared embryos by using SSR-markers, an existing genetic reference data set from the Northwest German Forest Research Institute (NW-FVA) archive was used to

characterize species specific genetic profiles. This reference material consisted of clearly morphologically determined trees (38 black walnut and 30 Persian walnut trees, four hybrids) from a seed orchard, a forest botanical garden and two nurseries in Germany (Table 1).

DNA fingerprinting

Lab procedures

After grinding the frozen embryo tissue in liquid nitrogen with a Mixer Mill (MM 200, Retsch), DNA was extracted using the protocol of Dumolin et al. (1995) based on CTAB/dichlormethane. DNA stock solutions were diluted to a final concentration of 10 ng/µL DNA and stored at −20 °C until further analyses. Six microsatellites (WGA027, WGA118, WGA089, WGA331, WGA069, WGA276; see Table 3) were amplified according to Pollegioni et al. (2009, 2012). PCR amplification fragments were resolved by capillary electrophoresis on a GeXP Genetic Analysis System (Beckman Coulter).

Analysis of SSR-data

Genetic variation was described using four population genetic parameters. We calculated the total and the effective number of alleles (N_A, N_E, respectively (Crow and Kimura 1970; Gregorius 1978), as well as the total number of private alleles (P) for each locus. Allelic profiles were presented on the basis of allele frequencies for the reference material (*J. nigra* and *J. regia*). The fixation index F (Hedrick 2011), indicating deviations from Hardy–Weinberg equilibrium, was calculated to estimate the risk of existing null alleles. The significance was tested using the Chi square-test.

In order to assign the individuals into different groups, two statistical approaches were used: the principle coordinate analysis displayed the pairwise (squared) Eucledian distances of the genotypes (Smouse and Peakall 1999) in a bidimensional plot using GenAlex software package, (Peakall and Smouse 2006) and the program STRUCTURE assigned the individuals into reproductive groups as to achieve conditions preferably close to Hardy–Weinberg equilibrium (within the groups; Pritchard et al. 2000a, b). The software package STRUCTURE 'harvester' calculated the most likely number of groups to which the different genotypes have to be assigned to (Earl and Von Holdt 2012; Evanno et al. 2005).

In vitro culture conditions and culture media

After the DNA-based hybrid identification, shoot cultures of the hybrid genotypes were cultured in order to compare two basal media (Duchefa, Harlem, The Netherlands), DKW medium (Driver and Kuniyuki 1984) and Rugini olive medium (Rugini 1984; Table 2). The cytokinin BA was applied in three concentrations: 2.2, 4.4, and 8.8 µM, each combined with 0.2 µM IBA. Glucose was used at 30 g L^{-1} and Kobe agar (Serva, Heidelberg, Germany) at 7 g L^{-1}. All media were sterilized by autoclaving at 121 °C and 0.1 MPa pressure for 20 min. The pH was adjusted to 5.7 prior to autoclaving. Cultures were maintained in a growth cabinet under a temperature of 27 ± 1 °C and a 16 h photoperiod supplied by cool white fluorescent lamps at 75 µmol m^{-2} s^{-1}.

After 21 days, stem segments of 0.3–0.5 cm length without callus were subcultured in 250 mL glass jars containing 80 mL of solidified medium. The callus fresh mass, the shoot fresh mass, the main shoot length, and the number of axillary buds were recorded at the end of the experiment (every 21 days). The experimental design was completely randomized. Each experiment was carried out twice (two successive subcultures); each subculture included seven replicates with five explants per replicate.

The data of the first subculture were not used for statistical analyses, in order to allow the cultures to adapt to

Table 1 Amount and origin of the reference material for genetic analyses

Source	Reference material			Putative hybrid embryos
	J. regia	*J. nigra*	Hybrids	
Seed orchard NW-FVA[a]	26	22	/	/
Forest botanical garden[b]	/	13	/	/
Nursery NW-FVA[c]	4	2	2	/
Private nursery[d]	/	1[e]	2	38
Total	30	38	4	38

[a] Seed orchard with *J. regia* and *J. nigra* in Oldendorf (Lower Saxony)

[b] Forest botanical garden in Hann. Münden (Lower Saxony)

[c] Nurseries of the NW-FVA (Northwest German Forest Research Institute)

[d] A private nursery in Southwest Germany (Anton Schott)

[e] The female parent of the tested embryo material

Table 2 Composition of the tissue culture media used for *Juglans* shoot multiplication

Nutrient	Culture medium			
	DKW		Rugini olive medium	
	(mg/L)	(mM)	(mg/L)	(mM)
Macronutrients				
NH_4NO_3	1416.00	17.70	412.00	5.15
$CaCl_2$	112.50	1.01	332.16	2.99
$Ca(NO_3)_2 \times 2H_2O$	1664.64	8.3	–	
$Ca(NO_3)_2$	–		416.92	2.54
KH_2PO_4	265.00	1.95	340.00	2.50
K_2SO_4	1559.00	8.95	–	
KNO_3	–		1100.00	10.88
$MgSO_4$	361.49	3.00	732.60	6.09
KCl	–		500.00	6.71
		(µM)		(µM)
Micronutrients				
H_3BO_3	4.80	77.63	12.40	200.55
$CuSO_4 \times 5H_2O$	0.25	1.00	0.25	1.00
$MnSO_4 \times H_2O$	33.80	200.00	16.90	100.00
$Na_2MoO_4 \times 2H_2O$	0.39	1.61	0.25	1.03
$ZnSO_4 \times 7H_2O$	17.00	59,12	14.30	49.75
KJ	–		0.83	5.00
$CoCl_2 \times 6H_2O$	–		0.025	0.11
FeNa EDTA	44.63	121.61	36.70	100.00
Vitamins and other organic compounds				
Nicotinic acid	1.00	8.12	5.00	40.62
Thiamine HCl	2.00	5.93	0.50	1.48
Pyridoxine HCl	–		0.50	2.43
Myo-inositol	100.00	554.94	100.00	554.94
Biotin	–		0.05	0,20
Folic acid	–		0.50	1.13
Glycine	2.00	26.64	2.00	26.64

Adapted from Driver and Kuniyuki (1984) and Rugini (1984)

the new media. The culture medium and BA concentration from the first experiment that resulted in the highest multiplication rate was used for the second experiment. In the multiplication phase, shoots of clone B22 were cultured on media with three different gelling agents: Gelrite (Trademark of Merck & Co., Inc. (Rahway, NJ) Kelco Division, USA): 3 g L^{-1}, Oxoid agar (Thermo Fisher Scientific): 7 g L^{-1}, and Kobe agar (Agar Kobe 1, Serva, Heidelberg, Germany): 7 g L^{-1}. The parameters evaluated were the same as in the experiment 1.

The main shoots being 2–4 cm in length of the second experiment were used for the rooting experiment. In the rooting experiments, two phases wereperformed, based on Jay-Allemand and Cornu (1986): (1) Induction phase: microcuttings were placed on root induction medium for 5 days at 27 °C in darkness. The induction medium was supplemented with 4, 12 and 20 µM IBA, respectively, 30 g L^{-1} sucrose, 7 g L^{-1} Kobe agar and the pH was adjusted to 5.7. Thereafter, they were transferred to the root expression medium (100 mL DKW gelified medium with macroelements diluted to ¼ (DGM) and 85 g vermiculite). (2) Expression phase: microcuttings produced with the optimal IBA concentration during the induction phase were used for the expression phase. The root expression media were supplemented with 30 g L^{-1} sucrose and the pH was adjusted to 5.7. Three root expression media without plant growth regulators were compared: (1) 100 mL DKW medium with DKW macroelements diluted to ¼ gelified by 2 g L^{-1} Gelrite (DGM) and 85 g vermiculite, (2) 100 mL DGM and 190 g sand (0.25–0.5 mm) and (3) 45 mL liquid DKW medium with macroelement diluted to ¼ (Fig. 4b). The experiment was completely randomized. Each experiment included six replicates with five explants per replicate. The parameters evaluated after 21 days were main root length and root number per rooted shoot.

For acclimatization, rooted and unrooted shoots (n = 30 per treatment) after the expression phase were washed thoroughly to remove residual medium and inserted directly in a mix of a commercial substrate and perlite 1:1 (Kleeschulte Profi-Linie Anzuchtsubstrat: www.kleeschulte-erden.de; Perlite: Perlite Dämmstoff GmbH) and placed in a greenhouse under an intermittent fog system. The commercial substrate contains white peat and bark humus to an unspecified amount. The humidity was 98 % from the 1st to the 14th day followed by 90 % from the 15th to the 21st day. The temperature was 24/21 °C (day/night). Plantlets were acclimatized in the greenhouse under natural light conditions. On sunny days shade screens with 50 % shading are closed automatically when the light intensity reaches 40,000 Lux. Plants were not fertilized during acclimatization.

Analysis of variance (ANOVA) or nonparametric ANOVA (Kruskal–Wallis testing) were calculated. Separation of means was obtained by using Tukey's HSD test ($p < 0.05$) (ANOVA) or multiple comparisons of mean ranks with p and z' values (nonparametric ANOVA) for all in vitro experiments was carried out using Statistica, Version 12.

Results

Hybrid identification by SSR fingerprinting

Genetic variation and allele profiles in the reference data set

In total, 94 alleles were detected in the whole reference data set at the six microsatellite loci. The genetic

variability of *J. nigra* turned out to be much higher than that of *J. regia* with 73 and 27 identified alleles, respectively (Table 3).

The microsatellite data indicated a high genetic distance between the two species. Three of the six microsatellites had no alleles in common (WGA027, WGA118, WGA331). Further, their allelic profiles showed no overlapping of the ranges of fragment lengths between the two species. At the remaining loci, an overlap of six alleles with identical fragment lengths could be observed (size homoplasy not tested; Fig. 1; Table 3).

Null alleles may also play a significant role in applying these loci for species determination. The fixation index (F) was significantly positive (homozygote excess) for the locus WGA276 in *J. nigra* and for the loci WGA331 and WGA069 in *J. regia*. The risk of existing null alleles may lead to detect false-homozygote individuals, particularly also in hybrid individuals which are expected to be heterozygous at most of their loci (Table 3).

Taxonomic identification of embryonic material

Both, the results of the principal component analysis as well as of the software STRUCTURE show that the two species *J. nigra* and *J. regia* and their hybrids (*J.* × *intermedia*) can unambiguously be determined by microsatellite marker data (Fig. 2). Further it turned out that almost 30 % of the analysed embryos from open pollination were identified to be hybrids *J. nigra* × *J. regia*.

The principle coordinate analysis (Fig. 2, upper diagramm), using a genetic distance parameter, arranges *J. regia*, *J. nigra* and *J.* × *intermedia* samples in three distinct groups. *J. regia* individuals are grouped in a smaller cluster on the left due to lower genetic variability than *J. nigra* (larger cluster on the right). *J.* × *intermedia* samples are clustered between them, sharing half of the genetic make-up of the two pure species.

The STRUCTURE method (Fig. 2, lower bar diagram), analysing mating conditions in a set of individuals, assign the individuals to two reproductive groups: the group of *J. nigra* and *J. regia* (dark and light grey bars). Hybrid individuals appear as divided bars (50 % dark and light grey) because of having received half of the genetic information from each of the two originally reproductive groups (*J. nigra* and *J. regia*).

This subdivision showed the highest likelyhood after conducting the software 'STRUCTURE harvester' according to Earl and Von Holdt (2012). In some "pure species" individuals however, there seems to be some variation that can be explained by the presence of common alleles of Black and Persian walnut within one genotype. Altogether, the used genetic methods allow an accurate hybrid determination in plant material of early ontogenetic stages.

In concordance with the four hybrids BAS 12-5 (B10), BAS 12-1 (B14), BAS 12-4 (B17), BAS 22-16 (B22) of the reference data set, eleven of the 38 embryos were identified to be *J. nigra* × *J. regia* hybrids, while the rest were *J. nigra* offsprings. They were assigned to both initial species with equal proportions (about 50 %) confirming their hybrid status.

Effect of the basal medium composition and BA concentrations on shoot multiplication

There were significant differences in the callus fresh mass produced at the shoot bases for the four clones after 21 days of culture among the three concentrations of BA ($p < 0.05$), except for the clone B17 on Rugini medium (Table 4). The higher BA concentration resulted in higher callus fresh mass. Callus fresh mass produced by 8.8 µM BA was highest on both DKW and Rugini medium for the clones B14 (0.9 and 1.7 g, respectively), B17 (1.4 and 1.8 g, respectively), and B22 (1.4 and 1.9 g, respectively). The only exception was clone B10 on DKW medium with the highest callus fresh mass produced by the lowest concentration of 2.2 µM BA (0.9 g), while on Rugini medium the highest callus fresh mass (1.17 g) was produced with 8.8 µM BA as in the other three clones.

A general tendency of decreasing shoot length with increasing BA concentrations was observed. The main shoot length of all four clones was highest on Rugini medium containing 2.2 µM BA (Fig. 3). On DKW medium the response was different, only clone B10 showed the

Table 3 Results of the nuclear simple sequence repeat (nSSR) marker analyses: the range of fragment lengths in base pairs, total and effective number of alleles (N_A, N_E, respectively) as well as the number of private alleles P for the reference data set of *J. regia* and *J. nigra*

SSR-locus	*J. nigra*						*J. regia*					
	bp.	N_A	N_E	P	F		bp.	N_A	N_E	P	F	
WGA027	211–243	14	5.18	14	0.09	n.s.	205–209	2	1.98	2	0.13	n.s.
WGA118	209–243	14	7.15	14	0.02	n.s.	185–207	4	1.93	4	0.03	n.s.
WGA089	186–232	14	9.76	13	0.09	n.s.	218–222	3	2.32	2	0.06	n.s.
WGA331	180–200	10	3.95	10	0.15	n.s.	272–276	3	2.06	3	0.55	***
WGA069	166–184	8	3.22	6	0.11	n.s.	160–182	6	3.18	4	0.22	**
WGA276	146–182	13	8.47	10	0.14	*	168–194	9	3.01	6	−0.15	**

*, **, *** The female parent of the tested embryo material

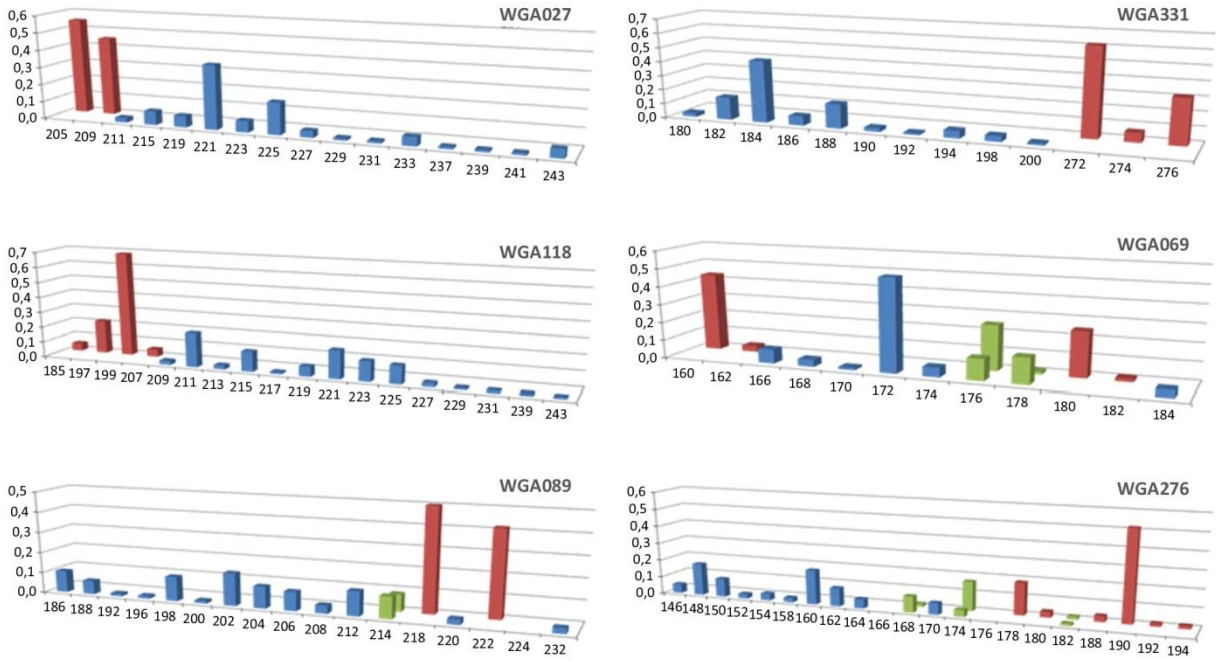

Fig. 1 Allele frequency profiles at the applied six microsatellite loci WGA027, WGA118, WGA089, WGA331, WGA069, WGA276; *blue bars* (*squares*): alleles specific for *J. nigra*; *red bars* (*squares*): alleles specific for *J. regia*; *green bars* (*squares*): alleles common for both species. X-axes: allele length in base pair; Y-axes: relative frequency of the allele. (Color figure online)

highest shoot length (4.9 cm) on the lowest BA concentration, whereas clone B17 had the highest shoots (3.7 cm) on 4.4 µM BA. For the clones B14 and B22 no significant differences in the main shoot length was detected on DKW medium among the different BA concentrations ($p < 0.05$; Table 4).

The highest number of nodes was observed in all clones when applying 8.8 µM BA in both, DKW and Rugini medium. The only exception was clone B17 on Rugini medium, where the highest number of axillary buds (9.1) was produced with 4.4 µM BA.

Response of Juglans hybrid clone B22′s explants to different gelling agents

Effects of the gelling agents on the callus fresh mass, the shoot fresh mass, the main shoot length, and the number of axillary buds of *Juglans* hybrid (Clone B22) after 21 days of culture are shown in Table 5. Significant differences in the callus fresh mass were found between Oxoid agar and Kobe agar compared to Gelrite, with the lower values on the two agar media. No statistically significant differences in the shoot fresh mass were observed among the three gelling agents. The highest main shoot length (3.0 and 3.3 cm) and the highest number of axillary buds (7.7 and 7.4) were produced by shoots cultured on Oxoid and on Kobe agar when compared to Gelrite (shoot length 2.3 cm, axillary buds 5.7).

Effects of different IBA concentrations applied during the root induction phase on rooting

The highest rooting percentage between 50 and 73 % of the four clones were obtained after root induction with 12 and 20 µM IBA (Table 6). The results concerning the longest main roots are inconsistent for the four clones. The genotype B14 developed the longest main root after induction with 20 µM IBA (3.9 cm), while B17 produced the longest roots after induction with 4 µM IBA (4 cm). No statistically significant differences in main root length were observed for B10 and B22 among the three IBA concentrations ($p < 0.05$).

In terms of root number per microcutting, there were no significant differences among genotypes B10, B14 and B17 for three IBA concentrations. The only significant difference was observed with clone B22 with the highest number of roots (2.5) induced by the highest IBA concentration.

Effects of root expression media on rooting of microcuttings

For all four genotypes the highest rooting percentages were observed on DGM with vermiculite with the highest value of 67 % obtained with the clones B10 and B17 (Table 7). The main root length of the four clones was almost the same on DGM with vermiculite and DGM with sand. Regarding root

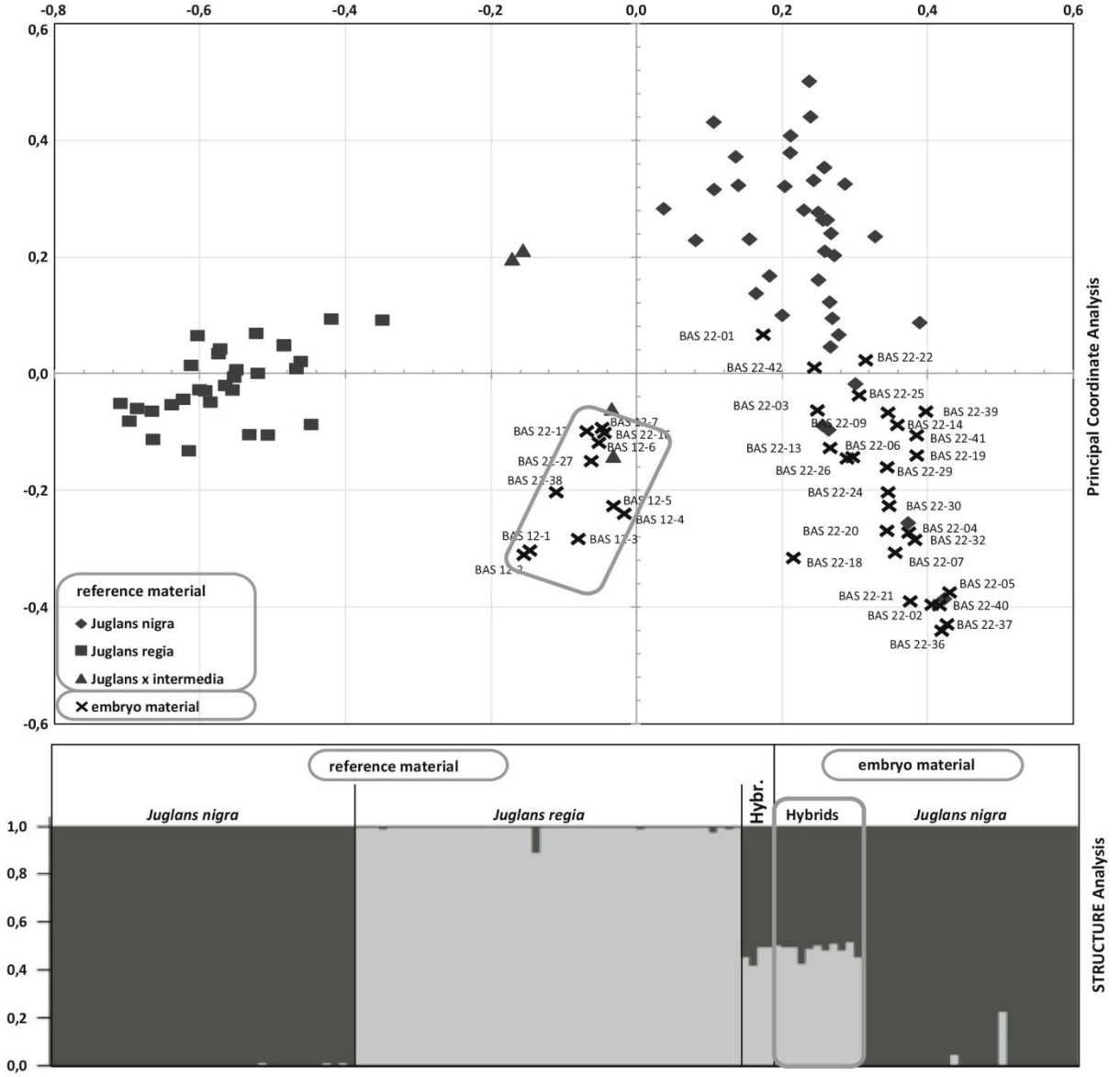

Fig. 2 Individual assignment of the genotypes into groups after application of the principle coordinate analysis (*upper diagram*) and STRUCTURE program (*lower diagram*)

number per microcutting, the highest values were found on DGM with vermiculite for clones B10 (2.5), B14 (2.6) and B22 (1.8), while shoots of B17 had produced the highest number of roots per microcutting on DGM with sand (3.5). However, the differences in the root number per microcutting were not significant between the two DGM treatments for the four clones ($p < 0.05$). In contrast to the two DGM treatments, no rooting was observed in liquid medium with the clones B10, B14 and B22. Only clone B17 showed a low rooting rate of 7 % in liquid medium (Fig. 4b, c).

During acclimatization in the greenhouse, 75–93 % of the plantlets of the four genotypes (B10, B14, B17 and B22) from root expression on DGM with vermiculite continued to grow vigorously (Table 7; Fig. 4d), whereas the plantlets from DGM with sand or liquid medium collapsed after transplanting to the greenhouse. More than 50 % of the unrooted plantlets with callus continued to grow, while the other plants with callus died immediately or survived only temporarily during acclimatization (21 days) without producing any further growth.

Table 4 Shoot cultures of four *Juglans* hybrid clones (n = 7 replicates of five shoots each) after 21 days of culture in different nutrient media and concentrations of BA. Beside BA the media contained 0.2 µM IBA

Clone	Nutrient medium	Concentrations of BA (µM)	Callus fresh mass (g)	Shoot fresh mass (g)	Main shoot length (cm)	No. axillary buds
B10	DKW	2.2	0.90 ± 0.21^{a}	0.33 ± 0.14^{a}	4.85 ± 1.54^{a}	8.66 ± 2.04^{a}
		4.4	0.87 ± 0.42^{a}	0.30 ± 0.11^{ab}	3.55 ± 1.41^{b}	8.20 ± 1.94^{a}
		8.8	0.63 ± 0.36^{b}	0.25 ± 0.08^{b}	1.70 ± 0.51^{c}	10.23 ± 2.57^{b}
	Rugini	2.2	0.55 ± 0.36^{a}	0.23 ± 0.08^{a}	3.38 ± 1.42^{a}	7.37 ± 1.00^{a}
		4.4	0.73 ± 0.15^{b}	0.35 ± 0.10^{b}	2.11 ± 0.76^{b}	10.26 ± 2.39^{b}
		8.8	1.17 ± 0.57^{c}	0.29 ± 0.13^{b}	0.98 ± 0.57^{c}	11.86 ± 2.83^{b}
B14	DKW	2.2	0.48 ± 0.19^{a}	0.29 ± 0.12^{a}	3.65 ± 1.36^{a}	7.20 ± 1.21^{a}
		4.4	0.57 ± 0.14^{a}	0.36 ± 0.83^{b}	4.10 ± 0.77^{a}	8.63 ± 1.17^{b}
		8.8	0.85 ± 0.29^{b}	0.38 ± 0.15^{b}	3.82 ± 0.92^{a}	10.94 ± 1.49^{c}
	Rugini	2.2	0.77 ± 0.41^{a}	0.32 ± 0.16^{a}	3.79 ± 1.77^{a}	8.63 ± 2.45^{a}
		4.4	1.47 ± 0.70^{b}	0.30 ± 0.22^{ab}	2.55 ± 1.32^{b}	7.11 ± 1.68^{b}
		8.8	1.74 ± 0.40^{b}	0.40 ± 0.15^{ac}	2.19 ± 0.71^{b}	9.49 ± 2.66^{a}
B17	DKW	2.2	0.53 ± 0.39^{a}	0.17 ± 0.08^{a}	1.89 ± 1.07^{a}	6.34 ± 2.07^{a}
		4.4	0.82 ± 0.50^{a}	0.32 ± 0.14^{b}	3.66 ± 1.27^{b}	8.57 ± 2.13^{b}
		8.8	1.41 ± 0.52^{b}	0.31 ± 0.15^{b}	2.89 ± 1.31^{c}	9.14 ± 2.02^{b}
	Rugini	2.2	1.82 ± 0.68^{a}	0.23 ± 0.14^{a}	3.19 ± 1.90^{a}	6.57 ± 2.66^{a}
		4.4	1.67 ± 0.53^{a}	0.20 ± 0.11^{ab}	2.06 ± 1.11^{a}	9.09 ± 2.51^{b}
		8.8	1.80 ± 0.58^{a}	0.15 ± 0.05^{b}	1.19 ± 0.50^{b}	7.03 ± 1.65^{a}
B22	DKW	2.2	0.63 ± 0.16^{a}	0.24 ± 0.09^{a}	3.09 ± 0.91^{a}	7.26 ± 1.22^{a}
		4.4	1.05 ± 0.29^{b}	0.30 ± 0.10^{b}	3.09 ± 0.59^{a}	8.46 ± 1.58^{b}
		8.8	1.41 ± 0.47^{c}	0.37 ± 0.16^{b}	2.88 ± 1.11^{a}	9.11 ± 3.26^{b}
	Rugini	2.2	1.27 ± 0.38^{a}	0.27 ± 0.14^{a}	3.34 ± 1.48^{a}	7.43 ± 2.54^{a}
		4.4	1.73 ± 0.39^{b}	0.35 ± 0.10^{b}	3.01 ± 1.11^{a}	8.60 ± 1.59^{ab}
		8.8	1.90 ± 0.46^{b}	0.35 ± 0.13^{b}	2.26 ± 0.81^{b}	9.37 ± 1.73^{b}

Values followed by the same letter within the same column which were evaluated by nonparametric ANOVA (Kruskal–Wallis testing) were not significantly different according to *p* and z' values. Comparisons were only done between BA concentrations within one genotype. Data are expressed as mean ± SD

Discussion

This study dealt with the optimization of micropropagation techniques for *Juglans* hybrids from embryonic tissue and used a DNA-based tool that allowed the detection of *Juglans* × *intermedia* genotypes in very early ontogenetic stages. In order to detect pure F1 hybrids and to exclude black walnuts arising from *J. nigra* trees as pollen donors, we first tested a set of microsatellites on unambiguously morphologically determined reference material (adult trees and seedlings) before using these markers for species and hybrid identification in seed material.

SSR markers allow non-ambiguous identification of hybrid embryos

We found that the six nSSR primer pairs (WGA027, WGA118, WGA089, WGA331, WGA069, WGA276) used to perform the DNA fingerprinting in this study efficiently discriminated *J. nigra* from *J. regia* genotypes and their interspecific hybrids (*J. nigra* × *J. regia*). Pollegioni et al. (2009) used ten microsatellite loci (WGA1, WGA4, WGA9, WGA69, WGA89, WGA118, WGA202, WGA276, WGA321, WGA331) for the genetic characterization of *J. nigra*, *J. regia* and *J.* × *intermedia*. Particularly, the nSSR locus WGA331 turned out to be very useful for species and hybrid identification due to a 100 bp insertion in *J. regia*. Altogether, the analysed microsatellites indicated high levels of genetic diversity. The much larger number of rare alleles in black walnut (*J. nigra*) compared to the Persian walnut (*J. regia*) becomes apparent in the number of effective alleles (allelic diversity) which is, over all loci, more than 2.5 times higher in *J. nigra* than in *J. regia* (Table 2; Pollegioni et al. 2009).

Efficient shoot multiplication on Rugini and DKW medium with 2.2 and 4.4 µM BA

Although DKW medium had been developed for Paradox rootstock (*J. hindsii* × *J. regia*), it is most widely used for walnut culture (Cornu and Jay-Allemand 1989; McGranahan

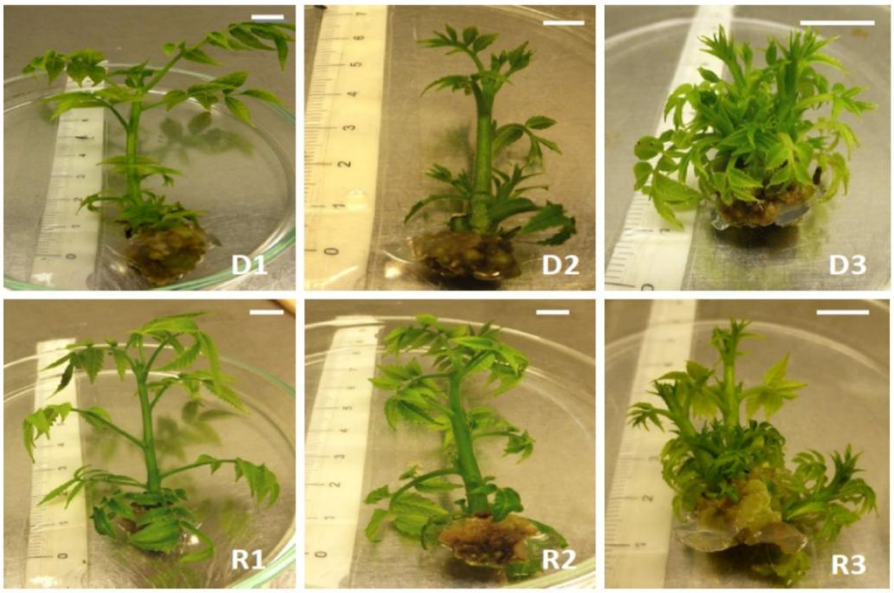

Fig. 3 Shoot culture of *Juglans* hybrid genotype B22 after 21 days of culture in different nutrient media and concentrations of BA (*bar* = 1 cm). D1 − 3: DKW medium with 2.2 µM BA + 0.2 µM IBA (D1), 4.4 µM BA + 0.2 µM IBA (D2) and 8.8 µM BA + 0.2 µM IBA (D3). R1 − 3: Rugini medium with 2.2 µM BA + 0.2 µM IBA(R1), 4.4 µM BA + 0.2 µM IBA (R2) and 8.8 µM BA + 0.2 µM IBA (R3)

Table 5 Effects of the gelling agents on the callus fresh mass, the shoot fresh mass, the main shoot length and the number of axillary buds of *Juglans* hybrid clone B22 explants (n = 7 replicates of five shoots each) after 21 days of culture

Gelling agent	Callus fresh mass (g)	Shoot fresh mass (g)	Main shoot length (cm)	No. axillary buds
Gelrite	0.89 ± 0.28^{a}	0.20 ± 0.24^{a}	2.28 ± 1.14^{a}	5.69 ± 2.03^{a}
Oxoid agar	0.62 ± 0.23^{b}	0.27 ± 0.18^{a}	2.95 ± 1.15^{b}	7.66 ± 1.55^{b}
Kobe agar	0.64 ± 0.25^{b}	0.24 ± 0.10^{a}	3.27 ± 1.25^{b}	7.43 ± 1.69^{b}

Values followed by the same letter within the same column are not significantly different according to Tukey's HSD test at $p < 0.05$. Data are expressed as mean ± SD

et al. 1987, 1988). The reason is that this medium has proven suitable for a variety of *Juglandaceae* species (McGranahan et al. 1987; Saadat and Hennerty 2002). Other culture media such as MS (Murashige and Skoog 1962) and Gamborget al. (1968) have been used for culturing *Juglans* species with varying degrees of success. In addition, WPM (woody plant medium) and NGE medium have been applied for walnut (Sánchez-Zamora et al. 2006). Rugini olive medium (Rugini 1984), has been reported to be efficient for a wide array of olive cultivars' micropropagation (Rama and Pontikis 1990; Rugini 1984). After achieving good results in micropropagation of *Populus* species at the Northwest German Forest Research Institute (NW-FVA) (unpublished data), Rugini medium was tested in this study for the four *Juglans* hybrid clones for the first time.

In the present study, it turned out that the effect of the tested BA concentrations on shoot elongation was different on the two basic media. Our results indicated that when using Rugini medium the four *Juglans* hybrids exhibited the best shoot elongation with 2.2 µM BA (Table 4; Fig. 4a). However, on DKW medium the effect of the BA concentration was not consistent. One clone developed the longest shoots also with 2.2 µM BA, another one with 4.4 µM BA, whereas in two clones there was no significant effect of the BA concentration on main shoot length. Furthermore, three of four *Juglans* hybrid clones developed the longest shoots on DKW medium, only one of them on Rugini medium (Table 4). In micropropagation, the different nutrient contents in the culture medium (Rugini medium contains more Ca^{2+} and K^{+} but less NH_4^{+} and SO_4^{2-} than DKW medium besides differences in microelements and vitamins) may have a significant effect on shoot growth and multiplication. This finding is consistent with a study carried out by Šedivá et al. (2013) who conducted an experiment with horse chestnut (*Aesculus hippocastanum* L.) and found that regeneration of new

Table 6 Effects of IBA concentrations during the induction phase (5 days in darkness at 27 °C) on in vitro rooting of microcuttings of four *Juglans* hybrid clones (n = 6 replicates of five microcuttings each) after the induction phase the shoots were transferred to root expression medium (vermiculite mixed with gelified medium)

Clones	Rooting medium (induction phase)	Rooting (%)	Main root length (cm)	Root number per rooted shoot
B10	4 µM IBA	27	3.34 ± 1.49[a]	1.87 ± 0.64[a]
	12 µM IBA	63	3.42 ± 0.57[a]	3.11 ± 1.79[a]
	20 µM IBA	53	2.79 ± 1.13[a]	2.18 ± 1.38[a]
B14	4 µM IBA	43	3.20 ± 1.29[a]	1.38 ± 0.65[a]
	12 µM IBA	50	2.39 ± 1.57[a]	2.80 ± 1.82[a]
	20 µM IBA	53	3.86 ± 1.05[b]	1.63 ± 0.72[a]
B17	4 µM IBA	63	4.02 ± 1.64[a]	4.21 ± 2.44[a]
	12 µM IBA	73	2.53 ± 0.74[b]	3.18 ± 1.33[a]
	20 µM IBA	73	3.16 ± 1.24[ab]	4.09 ± 2.04[a]
B22	4 µM IBA	30	3.22 ± 1.24[a]	1.22 ± 0.67[a]
	12 µM IBA	47	4.00 ± 1.13[a]	1.79 ± 0.58[ab]
	20 µM IBA	60	3.71 ± 1.09[a]	2.50 ± 1.25[b]

Evaluation by nonparametric ANOVA (Kruskal–Wallis testing). Values followed by the same letter within the same column are not significantly different according to p and z' values. Comparisons were only done between IBA concentrations within one genotype. Data are expressed as mean ± SD

Table 7 Effects of root expression medium on rooting after root induction with 12 µM IBA for 5 days in darkness for mircocuttings of the four *Juglans* hybrid clones (n = 6 replicates of five microcuttings each) after 21 days of culture

Clones	Rooting medium (expression phase)	Rooting (%)	Main root length (cm)	Root number per rooted shoot	Acclimatization (%)
B10	DGM with sand	10	4.13 ± 1.59[a]	1.33 ± 0.58[a]	0
	Liquid medium	0	0[b]	0[b]	0
	DGM with vermiculite	67	4.70 ± 1.22[a]	2.45 ± 1.19[a]	85
B14	DGM with sand	13	2.75 ± 1.00[a]	1.50 ± 0.58[a]	0
	Liquid medium	0	0[b]	0[b]	0
	DGM with vermiculite	53	2.99 ± 0.89[a]	2.56 ± 1.55[a]	75
B17	DGM with sand	13	3.93 ± 1.27[a]	3.50 ± 1.91[a]	0
	Liquid medium	7	0.80 ± 0.14[b]	1.50 ± 0.71[a]	0
	DGM with vermiculite	67	3.18 ± 1.10[a]	2.60 ± 2.35[a]	90
B22	DGM with sand	23	2.69 ± 1.31[a]	1.57 ± 0.79[a]	0
	Liquid medium	0	0[b]	0[b]	0
	DGM with vermiculite	50	2.63 ± 1.00[a]	1.80 ± 1.01[a]	93

Values followed by the same letter within the same column are not significantly different according to Tukey's HSD test at $p < 0.05$. Data are expressed as mean ± SD

shoots was significantly influenced by nutrient content in the culture media.

Our results also showed that the best BA concentration for shoot multiplication of three clones (B14, B17, B22) including main shoot length and number of axillary buds, was 4.4 µM BA with 0.2 µM IBA in DKW medium (Fig. 3). Although, the highest number of axillary buds was produced by the treatment with 8.8 µM BA and 0.2 µM IBA. In fact, this treatment was not suitable for shoot multiplication, rooting and acclimatization of the four *Juglans* hybrid genotypes in both media since shoots in this case were slowly growing tiny clusters which developed only leaves during the next subculture. Rodriguez et al. (1989) used the culture medium K (h) [the medium developed by Cheng (1975) for Douglas fir (*Pseudotsuga menziesii*)] mixed with different concentrations of BA (0, 4, 20 and 40 µM) and IBA (0, 0.4, 4 and 20 µM) for germination of *J. regia*. Their results indicated that the best treatment for

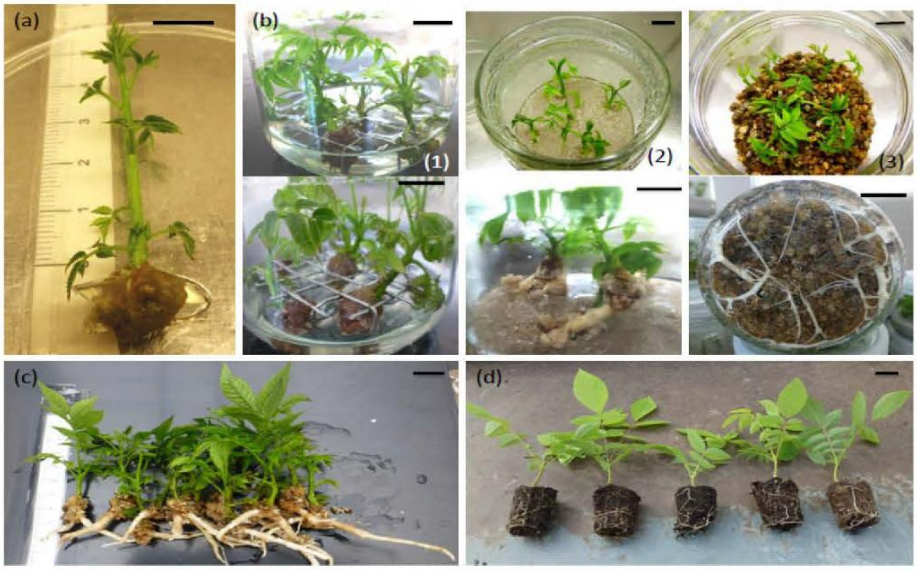

Fig. 4 Micropropagation of *Juglans* hybrids (*bar* = 1 cm). **a** Shoot culture of *Juglans* hybrid genotype B14 after 21 days of culture with 2.2 µM BA and 0.2 µM IBA in Rugini medium with Kobe agar, **b**-*1–3* different root expression systems in vitro: liquid medium (*b-1*); sand (*b-2*); vermiculite (*b-3*), **c** in vitro rooting of microcuttings of *Juglans* hybrid clone B17 with 12 µM IBA in the dark at 27 °C for 5 days followed by transfer to root expression medium with vermiculite, **d** plantlets in the greenhouse

multiple shoot bud formation was 40 µM BA without IBA. Furthermore, Revilla et al. (1989) reported that the best growth regulator treatment is 1 mg/l BAP and 0.1 mg/l IBA. Regarding to in vitro shoot multiplication of Persian walnut (*J. regia* L.) Saadat and Hennerty (2002) concluded that the optimal medium was DKW with 2.2 g/l phytagel, 1 mg/l BAP and 0.01 mg/l IBA. Furthermore, Bosela and Michler (2008) reported that the fastest elongation of black walnut (*J. nigra*) was observed on DKW medium with BA at a concentration of 5 µM. According to Driver and Kuniyuki (1984) who studied on Paradox Walnut (*Juglans hindsii* × *J. regia*), the optimum for multiple shoot development was 4.5 µM BA and 5 nM IBA in DKW medium. Our results also showed that the best BA concentration for the three clones' (B14, B17, B22) shoot multiplication including main shoot length and number of axillary buds, was 4.4 µM BA mixed with 0.2 µM IBA in DKW medium. This result is in accordance with the study of Driver and Kuniyuki (1984) on Paradox Walnut, and with Revilla et al. (1989), McGranahan et al. (1987) and Penuela et al. (1987) on *J. regia* microcuttings and with Bosela and Michler (2008) on black walnut (*J. nigra*). In the present study, each genotype reacted differently to the different BA concentrations and a significant interaction of the two factors genotype and BA concentrations was detected by ANOVA. The best medium for shoot elongation of three clones (B10, B14 and B17) included 2.2 µM BA and 0.2 µM IBA in Rugini medium.

Agar resulted in better shoot proliferation of Juglans hybrid clone B22 than Gelrite

The effects of different solidifying compounds on *Juglans* hybrid shoot cultures (Clone B22) indicated that a combination of Rugini medium with Kobe agar was optimal for this clone. The lowest callus fresh mass was recorded in Oxoid agar and significant differences in the callus fresh mass was found among Oxoid agar, Kobe agar and Gelrite. Kobe Agar is extracted from red algae such as *Gelidium* species or other seaweeds, while Oxoid agar is a complex mixture of polysaccharides extracted from red algae species. Since the in vitro response to different media always is genotype dependent, a comparison of these gelling agents in other genotypes should be tested in future experiments.

In the present study, the shoots for B22 genotype growing in Rugini medium with Kobe and Oxoid agar produced more buds and were higher than those cultivated on Gelrite (Table 5), these results are in disagreement with results reported by Barbas et al. (1993) and Saadat and Hennerty (2002) who concluded that Gelrite promoted shoot elongation while agar strongly inhibits elongation of explants in DKW medium. Furthermore, Cornu and Jay-Allemand (1989) also demonstrated that shoots on Difco Bacto agar added into DKW medium produced a lower number of buds, elongated less, but expressed more chlorosis than those on Gelrite in DKW medium. However,

21 days after culture on gelling agents in the Rugini medium the leaves of *Juglans* hybrid shoots were smaller than that of obtained on the Gelrite medium. On the other hand, the leaves formed on the Gelrite solidified medium were fresher and greener than those on media with Oxoid and Kobe agar. Barbas et al. (1993) observed similar effects and explained that Gelrite contain a higher amount of macroelements (Ca, Mg, and K) and iron (Fe) than agar, whereas a higher amount of Na and Cu was found in agar than in Gelrite. In this study, the difference in quantities (Gelrite with 3 g L^{-1}, Oxoid and Kobe agar with 7 g L^{-1}) requiring for medium solidification leaded to a greater amount of Na, P, Mn, Cu and B in media with agar than with Gelrite.

In our study, *Juglans* hybrid shoots grown in three kinds of gelling agents (Oxoid agar, Kobe agar and Gelrite) added to Rugini medium did not show any symptoms of hyperhydricity. However, there are reports of hyperhydricity when using DKW medium with Gelrite and agar: According to Leslie and McGranahan (1992) hyperhydricity was observed in walnut cultures when using DKW medium with agar, but not with Gelrite which is produced by microbial fermentation and is often used in plant tissue culture due to its high purity and the smaller quantity needed to obtain a hardness comparable to agar Huang et al. (1995).

In our lab at NW-FVA a long-term propagation rate of 3.0 after 5-weeks subcultures, which is suitable for commercial micropropagation, was achieved with a mature *Juglans* hybrid clone on a Rugini medium (Meier-Dinkel and Wenzlitschke 2016). The data of the mature clone were collected in a different way than described for the clones in this study. However, the obvious growth of the clones established from zygotic is faster and stronger than that of the mature clone, which results in a higher propagation rate than 3.0. In average, about 9–10 nodes were produced per explant at the end of the subculture. When assuming that new explants for propagation consist of two nodes this gives a calculated propagation rate of about 4.5. This can be explained with the more juvenile status of the clones in this study. Thus, a commercial feasible large-scale propagation can be established with the investigated hybrid clones. This is also supported by the obtained rooting rates between 50 and 73 % and the survival rates after acclimatization of 75–93 %.

Optimal root induction with 12 µM IBA applied for 5 days in darkness

In the present study, for genotype B17 the highest rooting percentage was obtained with microcuttings cultured on DKW medium containing 12 µM (Fig. 4c) and 20 µM IBA for 5 days in darkness. However, the shoot tips of four genotypes showed necroses after applying IBA at a concentration of 20 µM, and in consequence, the best rooting responses were obtained on DKW medium with 12 µM IBA for 5 days in darkness. Regarding the induction period, according to Jay-Allemand et al. (1992), the pretreatment of hybrid microcuttings (*J. nigra* × *J. regia*) with 24.6 µM IBA for 5 days in darkness is essential for root induction. As a result rooting rates were enhanced from 15 to 50 %, while Scaltsoyiannes et al. (1998), who used the same concentration of IBA in *J. regia*, found that optimum root induction was recorded after 6 days in darkness. They further proved that rooting depends on the genotype and the interaction of internal and external factors. Leslie and McGranahan (1992) reported that the highest rooting frequency (75 %) occurred on microshoots placed on half-strength MS medium containing 2.5 µM IBA for 7 days in darkness. Furthermore, Vahdati et al. (2004) concluded that the rooting percentage and root length in *J. regia* were not affected by the duration of the induction period.

Vermiculite promotes root development and growth

Regarding root expression media, there were significant differences in the four genotypes' rooting percentage ($p < 0.05$) with vermiculite and Gelrite being the best variant. When studying black walnut (*J. nigra*) rooting, Heile-Sudholt et al. (1986) demonstrated that the best rooting was observed in DKW medium with sterile vermiculite following a 15 s dip in 10 mM IBA. The same result was observed with hybrid walnut by Jay-Allemand et al. (1992), concluding that the semi-solid medium, combining vermiculite with Gelrite, is a reliable substrate for root expression. A possible explanation in this case could be that aeration was improved. Aeration also plays an important role in raspberry root development with foam substrates (Gebhardt 1985). Vermiculite mixed with Gelrite seems to provide a good balance between aeration and the availability of water (humidity) and nutrients, which promoted root initiation and development (Jay-Allemand et al. 1992; Fig. 4b-3).

Sand was less effective than vermiculite in our experiments. Microcuttings in sand produced mostly adventitious roots that were thick and white, without secondary root formation, and the roots emerged above the rooting medium surface (Fig. 4b-2). This points to a worse aeration in sand compared to vermiculite. The liquid medium treatment showed the lowest rates of rooting with only short roots developing for genotype B17. Most likely, the lack of oxygen in liquid media is a major limiting factor for rooting (Fig. 4b-1). The induction and extension of adventitious roots were also directly associated with oxygen uptake and activation of the alternative pathway in the

respiratory metabolism of Jerusalem artichoke (Hase 1987).

During the acclimatization in the greenhouse, more than 75 % of the plantlets of four *Juglans* hybrids genotypes rooted in vitro on DGM with vermiculite continued to grow vigorously (Fig. 4d). New leaves which seem to be an important morphological criterion in the survival of rooted microcuttings, were formed during the acclimatization and the youngest leaves became similar to those of field-grown plants. Moreover, more than 50 % of the unrooted plantlets with callus continued to grow (with new leaves and secondary root formation). Nevertheless, the plantlets rooted in vitro continued to grow faster and stronger, and developed more secondary roots in the greenhouse (Fig. 4d) than the unrooted plantlets with callus. The plantlets of four *Juglans* hybrid genotypes produced on DGM with sand and liquid medium did not survive in the greenhouse.

Summarizing the in vitro rooting experiments of the four investigated *Juglans* hybrid genotypes, the optimal root induction was achieved by culturing the microcuttings on DKW medium supplemented with 12 µM IBA for 5 days in darkness followed by root expression in the light on a mixture of DGM and vermiculite. Rooting success in vitro of the tested *Juglans* hybrids was almost 60 % on average (between 50 and 67 %, respectively), and, regarding acclimatization, more than 75 % of the plantlets from in vitro rooting continued to grow vigorously.

In conclusion, the combination of optimized in vitro propagation methods as well as a DNA-based tool for the early identification of hybrid seeds, provides an essential basis for the production of a multiclonal variety of *Juglans* hybrids to be used in forestry. Their quality will be tested on different ecological sites in field trials to evaluate their applicability and to meet the increasing demand of the forest sector for highly valuable walnut timber with a very limited availability so far.

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Manuscript 2.3

Endophytic bacterial communities in *in vitro* shoot cultures derived from embryonic tissue of hybrid walnut (*Juglans* x *intermedia*)

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ORIGINAL ARTICLE

Endophytic bacterial communities in in vitro shoot cultures derived from embryonic tissue of hybrid walnut (*Juglans* × *intermedia*)

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Abstract A detailed picture of the endophytic bacterial community structure on phylum and genus level in in vitro shoot cultures of sixteen *Juglans* genotypes including *Juglans regia, Juglans nigra* and mainly *Juglans* hybrids was generated by using culture-dependent and culture-independent methods. A total of eight endophytic bacterial strains with a high identity score (97–100%) were isolated from tissue culture shoot material derived from embryonic tissue of *Juglans* hybrids by the culture-dependent approach. By sequence comparison of a 16S rRNA fragment to the NCBI database, they were identified as *Acinetobacter, Brevundimonas, Moraxella, Pseudomonas*, and *Roseomonas* species. The culture-independent approach involved Illumina MiSeq 16S rRNA amplicon sequencing. Five different phyla (*Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes*, and *Proteobacteria*) were analyzed in more detail. The phylum *Proteobacteria* including genera such as *Brevundimonas* spp., *Acinetobacter* spp. and *Moraxella* spp. showed the highest relative abundance of endophytic bacteria. Thus, the results of the sequencing approach accorded well with those obtained in the culture-dependent approach. The abundance of *Moraxella* spp. was correlated with a good micropropagation in the *Juglans* genotypes under investigation, whereas, *Brevundimonas* spp. and *Acinetobacter* spp. were only detected in clones with medium propagation rates. Other bacterial endophytes known as plant growth promoting bacteria such as *Pseudomonas* spp., *Erwinia* spp., *Burkholderia* spp., *Pelomonas* spp., or *Sphingomonas* spp. were also found in in vitro cultures of a wide range of *Juglans* genotypes.

Keywords 16S rRNA amplicon sequencing · Bacterial endophytes · *Juglans* hybrids · *Juglans* × *intermedia* · Illumina MiSeq sequencing · In vitro propagation

Abbreviations

rRNA	Ribosomal RNA
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
NGS	Next generation sequencing
BA	Benzylaminopurine
EDTA	Ethylenediaminetetraacetic acid
IBA	Indole-3-butyric acid

Introduction

Juglans × *intermedia* (hybrid walnut) combines most of the positive features of both parental species (*Juglans nigra* ♀ and *J. regia* ♂) such as potential vegetative vigor, distinct disease resistance, good wood quality, strong apical dominance and tolerance to spring frost etc. (Cornu and Jay-Allemand 1989; Fady et al. 2003; Pollegioni et al. 2013). Although there is an increasing demand of the forest sector for highly valuable walnut timber, the availability of

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J.×*intermedia* plant material is very limited so far because of rare hybridization events under natural conditions due to phenological and genetic incompatibilities (Pollegioni et al. 2012). Over the last decades, only two sources of *Juglans* hybrids were available: Offspring of a *J. nigra* tree after spontaneous hybridization in South Germany (commercialized as RENI, Anton Schott nursery, Leiselheim) (Tuan et al. 2016) and two French hybrid varieties [commercialized as NG23 and NG38, French National Institute for Agricultural Research (INRA) (Becquey 1990)].

Micropropagation starting from embryo axes has been becoming an important technical achievement in the development and production of new *J.*×*intermedia* clones (Jay-Allemand and Cornu 1986; Revilla et al. 1989; Barbas et al. 1993). In addition, a simple and efficient DNA-based tool was designed for hybrid identification in early ontogenetic stages (seed material). The combination of these two methods provides an essential basis for the production of a multiclonal variety of *Juglans* hybrids through determination of genetically superior genotypes in clonal field trials by quality and growth performance (Tuan et al. 2016). Up to now, a set of 31 new genotypes of *J.*×*intermedia* was established in vitro from 2013 to 2015 at the Northwest German Forest Research Institute (NW-FVA). Furthermore, in vitro shoot cultures of eight *J. regia* and six *J. nigra* genotypes are available for comparative analyses.

Bringing clones on the market, economic aspects call for in vitro techniques ensuring continuously high multiplication rates. In our studies, however, a wide range of propagation and rooting rates were observed for different genotypes. Problems concerning in vitro growth could only partly be solved by optimization of culture media or other growth conditions.

An increasing number of studies provide clear indications of an impact of variations in endophytic bacterial compositions on growth parameters of tree species in vivo as well as on propagation success in vitro. Endophytic bacteria are not only incorporated into plant tissues during plant growth through the root system or stomata etc., but can also be present in the seeds and thereby be introduced to the embryonic tissue culture (Cankar et al. 2005; Ulrich et al. 2008a; Scherling et al. 2009). Thus, a plausible explanation for difficulties in in vitro propagation could be seen in variations in endophytic bacterial populations.

Endophytes are bacteria or fungi that colonize the internal plant tissue without causing any symptoms of disease (Petrini 1991). According to Compant et al. (2005) possible beneficial effects of endophytic bacteria are plant growth promotion via supply of nutrients, pathogen inhibition and manipulation of plant growth by alteration of the phytohormone status. Several studies detected bacterial endophytes to have positive effects on woody plants, for instance for *Populus, Larix* and *Pinus* (Shishido et al. 1995; Bal 2003; Moore et al. 2006; Izumi et al. 2008; Ulrich et al. 2008b). Additionally, endophytes in tissue cultures of woody plants have also been associated with plant growth promotion, e.g. for *Prunus cerasus, Pinus sylvestris, Picea abies, Robinia pseudoacacia,* and *Prunus avium* (Kamoun et al. 1997; Laukkanen et al. 2000; Pirttilä et al. 2000; Cankar et al. 2005; Ulrich et al. 2008a; Quambusch et al. 2014). On the other hand, the bacterial endophytes can also have harmful effects on plants. Herman (1989) pointed out that non-pathogenic or plant growth-promoting bacteria can become harmful for plant tissues in micropropagation. An example are *Mycobacterium* species which, on the one hand, have been described as commensal or symbiotic endophytes of wheat (Conn and Franco 2004), rice (Mano et al. 2007), Scots pine (Pirttilä et al. 2004) and rock plant (Koskimäki et al. 2010). On the other hand, Laukkanen et al. (2000) and Quambusch et al. (2014) reached the conclusion that *Mycobacterium* spp. might have a negative influence during tissue culture of Scots pine (*P. sylvestris*) and *P. avium*, respectively.

Next generation sequencing (NGS) techniques have revolutionized molecular biology and the detection of microbial communities in particular (Oberauner et al. 2013; Uroz et al. 2013; Hong et al. 2015). The endophytic bacterial communities in micropropagated material of *Juglans* species have not been studied so far. Therefore, the aims of this study were (1) to analyze the bacterial population structure of in vitro shoot cultures of the three different *Juglans* groups, including eleven *J.*×*intermedia* (*J. nigra*×*J. regia regia)* genotypes, two *J. regia* genotypes and three *J. nigra* genotypes by both culture-dependent and culture-independent methods, and (2) to observe the differences between the bacterial populations of genotypes with differing propagation success in vitro for a first assessment of potential growth promoting or inhibiting bacterial strains.

Materials and methods

In vitro cultures

Shoot cultures of eleven *J.*×*intermedia* genotypes as well as two *J. regia* and three *J. nigra* genotypes (Table 1) were cultivated on DKW medium (Driver and Kuniyuki 1984) using the in vitro protocols according to Tuan et al. (2016). The numbers 13, 14 and 15 within the sample labels indicate the year of nut harvest. All hybrids and two *J. nigra* genotypes were derived from a single hybridizing black walnut tree of a nursery in South Germany (Anton Schott, Leiselheim), while the material of *J. regia* and one *J. nigra* genotype was collected in Hann. Münden, Germany (Table 1). The taxonomical status of hybrids and pure

Table 1 The origin of the *Juglans* genotypes material for analyses

Genotype	Species	Establishment	Harvest location
JH13-1	*Juglans* hybrid	4.12.2013	Kaiserstuhl
JH13-2	*Juglans* hybrid	4.12.2013	Kaiserstuhl
JH13-3	*Juglans* hybrid	4.12.2013	Kaiserstuhl
JH13-4	*Juglans* hybrid	4.12.2013	Kaiserstuhl
JH14-1	*Juglans* hybrid	14.10.2014	Kaiserstuhl
JH14-2	*Juglans* hybrid	14.10.2014	Kaiserstuhl
JH14-3	*Juglans* hybrid	14.10.2014	Kaiserstuhl
JH14-4	*Juglans* hybrid	14.10.2014	Kaiserstuhl
JH15-1	*Juglans* hybrid	19.10.2015	Kaiserstuhl
JH15-2	*Juglans* hybrid	19.10.2015	Kaiserstuhl
JH15-3	*Juglans* hybrid	19.10.2015	Kaiserstuhl
JR13-1	*Juglans regia*	7.11.2013	Hann. Münden
JR13-2	*Juglans regia*	8.11.2013	Hann. Münden
JN15-1	*Juglans nigra*	19.10.2015	Kaiserstuhl
JN15-2	*Juglans nigra*	19.10.2015	Kaiserstuhl
JN15-3	*Juglans nigra*	21.09.2015	Hann. Münden

species was identified by using SSR markers (Tuan et al. 2016).

The used DKW medium (Driver and Kuniyuki 1984) contained 30 gl^{-1} glucose and 7 gl^{-1} Kobe agar (Serva, Heidelberg, Germany). The medium was supplemented with 4.4 µM benzylaminopurine (BA) and 0.2 µM indole-3-butyric acid (IBA). All media were sterilized by autoclaving at 121 °C and 1.0 kg cm^{-2} pressure for 20 min. The pH was adjusted to 5.7 before autoclaving. Cultures were maintained in a growth cabinet at a temperature of 27 ± 1 °C and a 16 h photoperiod. The light was supplied by cool white fluorescent lamps with 75 µmol m^{-2} s^{-1}. At each of five successive subcultures seven replicates (250 ml glass jars containing 80 ml of solidified medium) with five explants per replicate were evaluated for the propagation rate. The propagation rate was defined as the number of new subculturable explants (shoot tips or segments with two leaves) at the end of the 21-days culture passage divided by the initial number of explants per vessel (Tuan et al. 2016).

Isolation and cultivation of bacteria

Three leaf or stem pieces of in vitro shoot cultures of all 16 *Juglans* genotypes were placed on plates with bacterial nutrient agar (R2A) medium (Carl Roth Co., Karlsruhe, Germany) and medium 523 (Viss et al. 1991) with three replicates per medium and genotype. This experiment was repeated twice. Afterwards, these plates were sealed with Parafilm®, incubated at 25 °C in the dark and monitored for bacterial growth for 5 weeks. Bacterial growth was observed at the explants cut surfaces (Fig. 2) and bacterial colonies were selected based on different color, shape and growth rate. Isolates were purified by re-streaking single colonies twice onto a fresh medium before storage and further analysis.

DNA extraction

For the extraction of genomic DNA of the cultivated bacteria, a loop full of bacteria was collected from a single colony and transferred to an Eppendorf tube containing 50 µl of lysozyme working solution (500 µg ml^{-} in Tris-EDTA) mixed thoroughly and incubated for 30 min at 37 °C. 10 µl of Proteinase K stock solution (20 mg ml^{-1}) and 20 µl of RNAse (10 mg ml^{-1}) were added and this mixture was again incubated for 60 min at 55 °C. Based on a modified protocol for DNA extraction from plant material published by Dumolin et al. (1995), the samples were mixed with 400 µl of dichloromethane and kept on ice for 10 min. After 5 min centrifugation at 4 °C (9838×*g*), the supernatant was transferred to a fresh tube and the DNA was precipitated with 400 µl of isopropanol, and stored overnight at −20 °C. After centrifugation for 10 min at 4 °C (16,627×*g*) the supernatant was discarded. Finally, the DNA pellet was washed with 70% ethanol and re-suspended in 100 µl RNase- and DNase-free water. The concentration and quality was analyzed using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Massachusetts, USA) and the DNA was stored at −20 °C.

For the amplicon sequencing approach (culture-independent approach), the total DNA of in vitro plant material including bacterial DNA was extracted from approximately 150–250 mg of plant material. Each sample included leaves, stems and shoot tips of three microshoots and was frozen in liquid nitrogen. The DNA extraction and purification was performed using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol.

Sanger-sequencing of isolated bacterial DNA

The 16S rRNA of the bacterial isolates was amplified using the primers 27f (AGAGTTTGATCCTGGCTCAG) and 1492r-Y (GGYTACCTTGTTACGACTT) (Weisburg et al. 1991) following the protocol by Quambusch et al. (2014). The PCR products of three parallel reactions were pooled before electrophoresis, and the bacterial amplification product of ~1500 bp was excised from the agarose gel. The PCR products were purified using the Nucleo Spin Gel and PCR clean-up kit (Macherey–Nagel, Düren, Germany), and sequenced by Seqlab (Göttingen, Germany) using primers 27f and 1492r-Y to obtain a nearly full-length sequence of 16S rRNA. The obtained sequences were combined using the CLC software (CLC bio, Aarhus, Denmark) and aligned against sequences

available in the NCBI GenBank database using BLAST (Basic Local Alignment Search Tool). The nucleotide sequence data of the isolates have been deposited in the GenBank database under the following accession numbers: KY501219-KY501220-KY501221-KY501222-KY501223-KY501224-KY501225-KY501226.

Sequencing of bacterial 16S rRNA with Illumina MiSeq

The samples for MiSeq sequencing were harvested from the same subculture stage for all genotypes and the analysis of the bacterial communities in 16 micropropagated *Juglans* genotypes was performed by LGC (LGC Genomics GmbH, Berlin, Germany). One genotype (JH 13-3) was collected in two consecutive years. The samples were analyzed in triplicate giving a total of 51 samples. The amplification and sequencing of the 16S bacterial ribosomal genes were carried out with the Illumina MiSeq system. The V3–V4 regions of the bacterial 16S rRNA gene were amplified by PCR using the combination of forward primer 799f (AACMGGATTAGATACCCKG) and reverse primer 1115r (AGGGTTGCGCTCGTTRC) using the company's protocol. Primer 799f (Chelius and Triplett 2001) was selected to preferentially amplify bacterial sequences and reduce the amount of chloroplast and mitochondrial PCR products.

Based on the protocol from LGC (LGC Genomics GmbH, Berlin, Germany), pre-processing and analyses of the raw sequencing data (De-multiplexing) of all libraries for each sequencing lane were conducted using the 1.8.4 version of the Illumina bcl2fastq conversion software.

The 16S pre-processing and operational taxonomic unit (OTU) clustering from amplicons were performed with the program Mothur 1.35.1 (Schloss et al. 2009) as follows: (i) removal of sequences containing ambiguous bases, with homopolymer stretches of more than 8 bases or with an average Phred quality score below 33; (ii) alignment against the 16S Mothur-Silva SEED r119 reference alignment; (iii) filtering of short alignments (truncated or unspecific PCR products), (iv) sequence subsampling to 50,000 sequences per sample; (v) sequencing error reduction by pre-clustering (up to 1 differing base per 100 bases allowed in a cluster) (vi) elimination of chimera with the uchime algorithm; (vii) taxonomical classification of the sequences (against the Silva reference classification) and removal of sequences from other domains of life; (viii) OTU picking by clustering at the 97% identity level (using the cluster.split method); (ix) OTU consensus taxonomical calling, integrating the taxonomical classification of the cluster member sequences; (x) finally, creation of OTU count tables.

Statistical and phylogenetic analyses

All sequences of the obtained isolates and reference sequences were aligned using the CLC software, and the regions with gaps were removed manually. A dendrogram was constructed using the neighbor-joining method by the CLC software, and statistical significance of nodes was determined by bootstrap analysis with 1000 repeats.

The rarefaction analysis based on Mothur 1.35.1 (Schloss et al. 2009) including the Chao and Shannon diversity indices was performed to reveal the diversity indices with 97% 16S rRNA gene sequence similarity by PAST 3 (3.02). For statistical test, we used the Dell Statistica version 12 (Tulsa, USA). The separation of means in propagation rates of eleven *J.*×*intermedia* genotypes was obtained by using multiple comparisons of mean ranks with $p < 0.05$ (nonparametric ANOVA). The relative abundances (read count per total of reads) of each OTU in different *Juglans* genotypes were obtained from transformed data which were checked by nonparametric ANOVA at $p < 0.05$.

Results

Propagation rates of 11 *Juglans* hybrid genotypes

Eleven *J.*×*intermedia* genotypes with different propagation rates were selected for this study. Five genotypes JH13-1, JH13-3, JH13-4, JH15-1 and JH 14-1 were easy to propagate (propagation rates >4, marked by +) with propagation rates of 4.2 up to 5.4 (Fig. 1; Supplement S1). The propagation rates for genotypes JH14-3, JH14-4, JH14-2, JH15-3, JH13-2, and JH15-2 were lower (propagation rates <4, marked by −) and ranged between 2.5 and 3.4 (Fig. 1). Due to the high standard deviations that reflect fluctuations over the five culture passages, only the propagation rates of the two best genotypes (JH13-1 and JH13-3) were significantly different from those of the group with the medium proliferation.

The micropropagation protocol was the same for all *Juglans* hybrids genotypes described here, but massive outgrowth of bacteria could only be observed for one genotype (JH13-2) during routine micropropagation.

Identification of endophytic bacteria in a culture-dependent approach

A total of nine bacterial strains was isolated from shoot cultures of six *Juglans* genotypes (Table 2). No bacteria were isolated from the other five *Juglans* hybrid genotypes, from three *J. nigra* and from two *J. regia* genotypes within 5 weeks of observation. Eight strains could be identified on the genus level and due to high identity scores of 99–100%

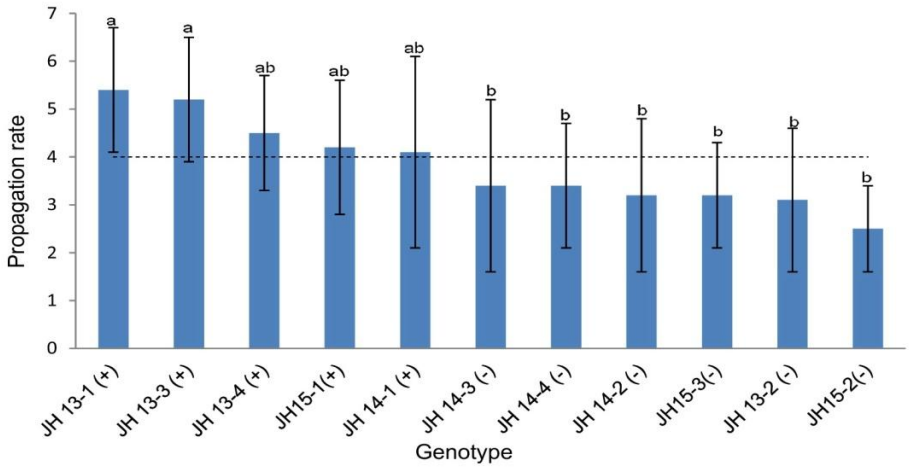

Fig. 1 Propagation rates of 11 *Juglans* hybrid genotypes averaged over five subcultures. Given are means and standard deviations of propagation rates taken at five subsequent subcultures, each including seven replicates with five shoots. (+) designates clones with a high propagation rates ≥4, (−) designates clones with medium propagation rates <4. Significant differences ($p < 0.05$) between propagation rates were indicated by *different letters*, after nonparametric ANOVA (Kruskal–Wallis test)

Table 2 Classification of endophytic bacteria isolated from in vitro shoot cultures of six *Juglans* hybrid genotypes using 16S rRNA sequence fragments

Isolate no.	Genotype	Genus	Closest match at NCBI[a]	Sequence length (bp)	% Identity
1	JH13-2 (−)	*Brevundimonas*	*Brevundimonas vesicularis*	1342	99.93
2	JH13-2 (−)	*Acinetobacter*	*Acinetobacter calcoaceticus*	1401	100
3	JH13-3 (+)	*Moraxella*	*Moraxella osloensis*	1382	99.71
4	JH13-4 (+)	*Moraxella*	*Moraxella osloensis*	1392	99.71
5	JH14-2 (−)	*Acinetobacter*	*Acinetobacter calcoaceticus*	1402	99.86
6	JH14-2 (−)	Isolate not identified	Isolate not identified	×	×
7	JH14-3 (−)	*Pseudomonas*	*Pseudomonas psychrotolerans*	1399	100
8	JH14-3 (−)	*Roseomonas*	*Roseomonas terpenica*	1332	99.92
9	JH14-4 (−)	*Acinetobacter*	*Acinetobacter calcoaceticus*	1401	100

[a]Closest match with description on species level

the 16S rRNA sequence allowed approximation of the species corresponding to the closest NCBI database entries (Table 2). One isolate could not be identified, because the sequencing was not successful, although the sample was sent for sequencing three times.

Bacteria isolated from the genotypes JH13-3 and JH13-4 with good propagation rates (+) formed white and slow growing colonies with 1 mm diameter after 1 week (Figs. 2, 3 c, d). The 16S rRNA of these isolates showed 99.71% sequence similarity to *Moraxella osloensis* (GenBank acc. number KF891343). Isolates from the four *Juglans* hybrid genotypes with medium propagation rate showed the highest sequence similarities with the genera *Brevundimonas, Acinetobacter, Pseudomonas* and *Roseomonas* (Table 2). From genotype JH13-2 two strains were derived, the first one formed dark yellow, slow-growing colonies after 3 days and presented the highest sequence similarity to *Brevundimonas vesicularis* (KU844054) (Figs. 2, 3a). The bacterial colonies of the second isolate could only be observed after 5 weeks and were identified as *Acinetobacter calcoaceticus* (KT229742). This bacterium was also detected in the genotypes JH14-2 and JH14-4. Furthermore, a second bacterium was isolated from genotype JH14-2, however, the isolated strain could not be identified. Two isolates were obtained from *Juglans* hybrid genotype JH14-3 and identified as *Pseudomonas psychrotolerans* (JQ659493) and *Roseomonas terpenica* (AM 503920) (Figs. 2, 3f, g).

Strikingly, all identified bacterial isolates belonged to either the *Alpha-* or the *Gammaproteobacteria* as can be seen in the dendrogram (Fig. 4). The composition of bacteria detected by the culture-dependent approach were different between the plant genotypes. However, some bacterial species such as *Acinetobacter calsoaceticus* were detected in up to three genotypes and the strains were shown to be highly related or identical, as illustrated by their clustering in the dendrogram (Fig. 4).

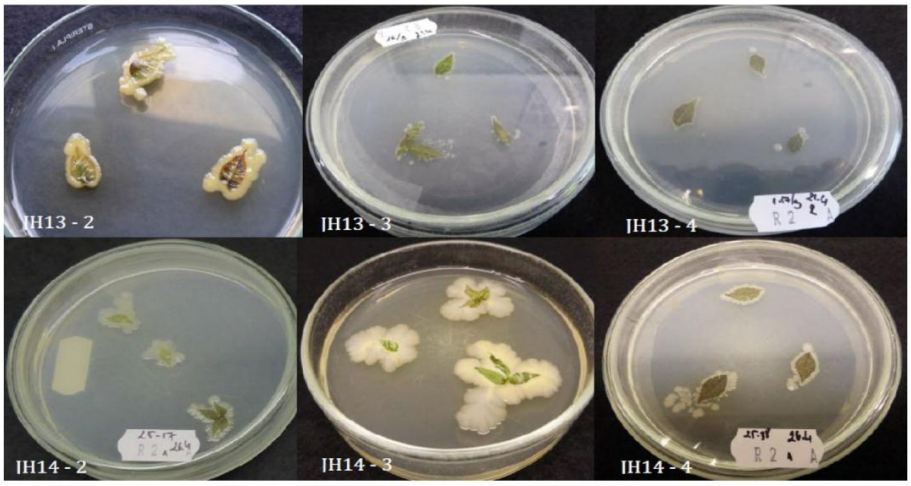

Fig. 2 Bacteria outgrowing from from in vitro leaves and stems of six *Juglans* hybrid genotypes cultured on bacterial nutrient agar (R2A) medium in 5 weeks (9 cm Petri dishes)

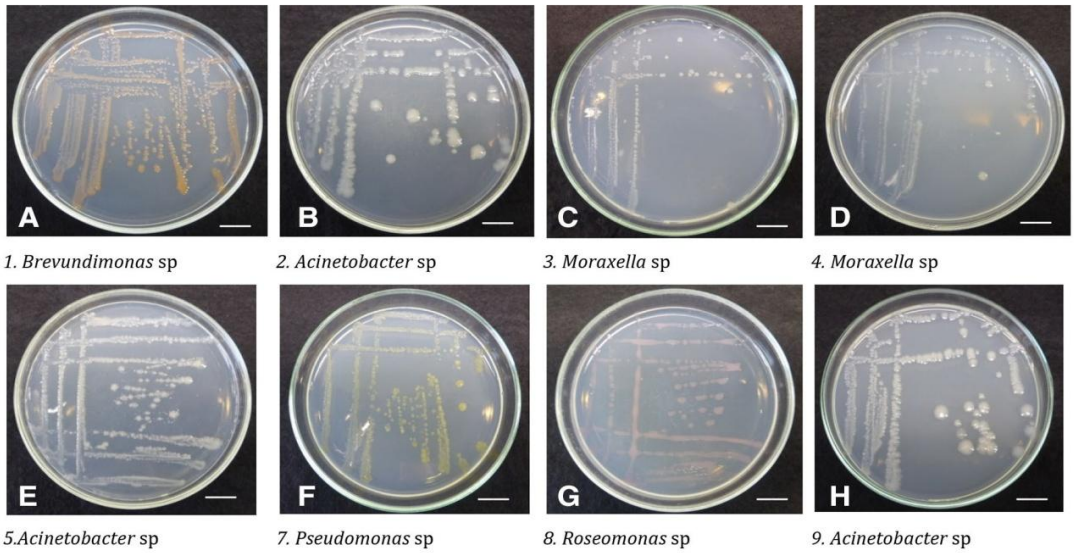

Fig. 3 Bacterial isolates obtained from in vitro leaves and stems of six *Juglans* hybrid genotypes cultured on medium R2A (*Bar* = 1 cm). The bacterial strains were isolated from JH13-2 (**a, b**); JH13-3 (**c**); JH13-4 (**d**); JH14-2 (**e**); JH14-3 (**f, g**); JH14-4 (**h**)

Culture-independent approach to reveal endophytic bacterial communities

Sequencing results and diversity indices

A total number of 80,577 reads and 1065 operational taxonomic units (OTUs) were obtained from 51 samples belonging to seventeen libraries through Illumina MiSeq sequencing analysis. The samples of each library contained an average of 44–8115 reads, with a number of different phylogenetic OTUs ranging from 15 to 32 (Table 3) and a read length of 300 bp for the majority of the samples.

Comparing the three *Juglans* species there were noticeable differences in the numbers of reads: While *J. nigra* genotypes (JN15-1, JN15-2, and JN15-3) had low numbers of reads (an average of 53, 47, and 44 reads within the library, respectively) the *Juglans* hybrid genotypes partly attained high and partly also low numbers of reads (Table 3). The two *J. regia* genotypes reached relatively high numbers of reads (691 and 1323) in average per library. All samples

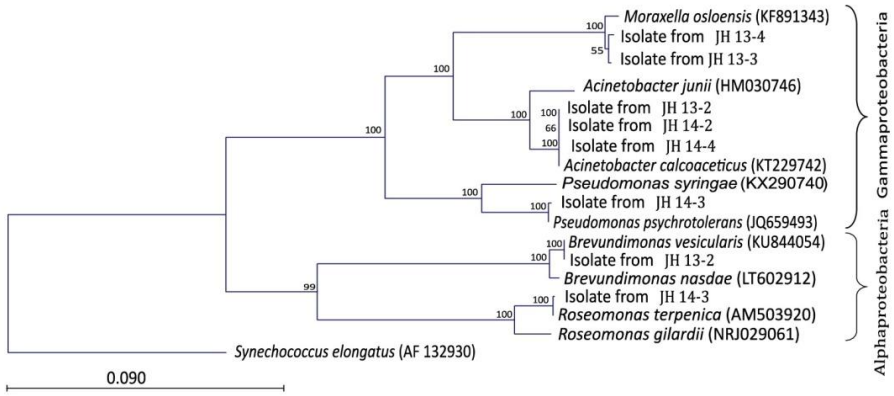

Fig. 4 Dendrogram based on the 16S rRNA fragment from bacterial isolates of different micropropagated *Juglans* hybrid genotypes compared to reference sequences (the closest hit according to BLASTn analysis and one sequence of an additional species for each genus). The dendrogram was inferred using the neighbor-joining analysis and *Synechococcus elongatus* was used as the outgroup. Values from 1000 bootstrap repeats are presented if support is >50%. The *scale bar* represents genetic differences based on the Jukes–Cantor correction. Positions containing gaps were eliminated from the dataset. *Numbers in parentheses* represent the sequence accession numbers in EMBL/GenBank

Table 3 Sequencing results and diversity estimates for in vitro shoot cultures of 16 *Juglans* genotypes using 16S rRNA sequences and Illumina MiSeq sequencing technology

Genotypes	Sequencing results		Diversity estimates[a]	
	Number of reads	Number of OTUs	Chao-1	Shannon
JH13-1	57 ± 21	18 ± 3	42.8 ± 17.5	2.2 ± 0.3
JH13-2	7633 ± 2054	27 ± 6	44.6 ± 12.4	0.8 ± 0.0
JH13-3 (I)	1498 ± 2355	24 ± 5	50.5 ± 43.6	0.6 ± 0.1
JH13-3 (II)	450 ± 258	21 ± 9	30.2 ± 10.6	0.6 ± 0.1
JH13-4	158 ± 87	19 ± 4	22.0 ± 10.3	1.3 ± 0.2
JH14-1	61 ± 14	22 ± 4	35.0 ± 15.1	2.5 ± 0.3
JH14-2	8115 ± 6230	21 ± 7	36.8 ± 10.6	1.0 ± 0.0
JH14-3	147 ± 83	32 ± 5	67.0 ± 13.9	2.5 ± 0.2
JH14-4	6341 ± 2630	23 ± 8	44.5 ± 12.4	0.1 ± 0.0
JH15-1	131 ± 89	27 ± 15	76.0 ± 16.6	2.4 ± 0.2
JH15-2	51 ± 20	19 ± 6	25.0 ± 11.1	2.2 ± 0.2
JH15-3	61 ± 14	20 ± 5	40.5 ± 17.6	2.4 ± 0.3
JR13-1	1323 ± 2194	19 ± 7	51.3 ± 17.1	0.3 ± 0.1
JR13-2	691 ± 555	15 ± 3	25.3 ± 12.0	0.4 ± 0.1
JN15-1	53 ± 46	18 ± 6	22.1 ± 11.1	2.1 ± 0.3
JN15-2	47 ± 33	17 ± 8	39.0 ± 14.0	2.1 ± 0.3
JN15-3	44 ± 25	16 ± 5	82.0 ± 16.8	2.1 ± 0.3

[a]*Chao-1*: Chao-1 species richness estimator, *Shannon*: Shannon-Weiner Index; a 97% similarity threshold was used to define operational taxonomic units (OTUs)

contained a high number of reads assigned to chloroplast (54.8–88.4%) or mitochondrial (7.0–30.5%) origin.

The diversity of species richness (Chao-1), and Shannon index indicate differences in the diversity of the bacterial population of the three *Juglans* groups (Table 3). However, they are strongly influenced by the total number of sequence reads and will therefore not be considered in detail, here. The number of different OTUs identified varied between 15 and 32 with similar numbers in the two parental species, and slightly higher numbers in some of the hybrids (JH14-3, JH13-2, JH15-1).

Taxonomic composition of endophytic bacterial communities in 16 Juglans genotypes

A total of 995 OTUs (Supplement S2) from 11 phyla, 20 classes, 51 orders, 92 families, and 160 genera were identified. Five different phyla (*Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes*, and *Proteobacteria*) were common to the sixteen libraries, and they made a contribution for more than 85% of the total reads in every library (Fig. 5a). *Proteobacteria*, accounting for 243 of the OTUs and 75.4% of the reads for all *Juglans* samples, was the most abundant group, followed by *Actinobacteria* (57 OTUs contributing for 17.8% of the reads) and *Firmicutes* (47 OTUs, accounting for 1.9% of reads) (Fig. 5a). 0.2% of the reads could not be classified.

The data (Fig. 5a; Supplement S3) also demonstrate that the proportion of *Proteobacteria* in the 16 *Juglans* samples showed a high variability with a range of 8.9% (JR13-2) to 99.3% (JH14-4). Moreover, the two most dominant classes in the *Proteobacteria* phylum, *Alphaproteobacteria* and *Gammaproteobacteria*, occupied 30.8 and 44.0% of all 16 *Juglans* genotypes, respectively. Again the proportions of the different classes varied between the genotypes, with some genotypes like JH13-3 and JH14-4 being

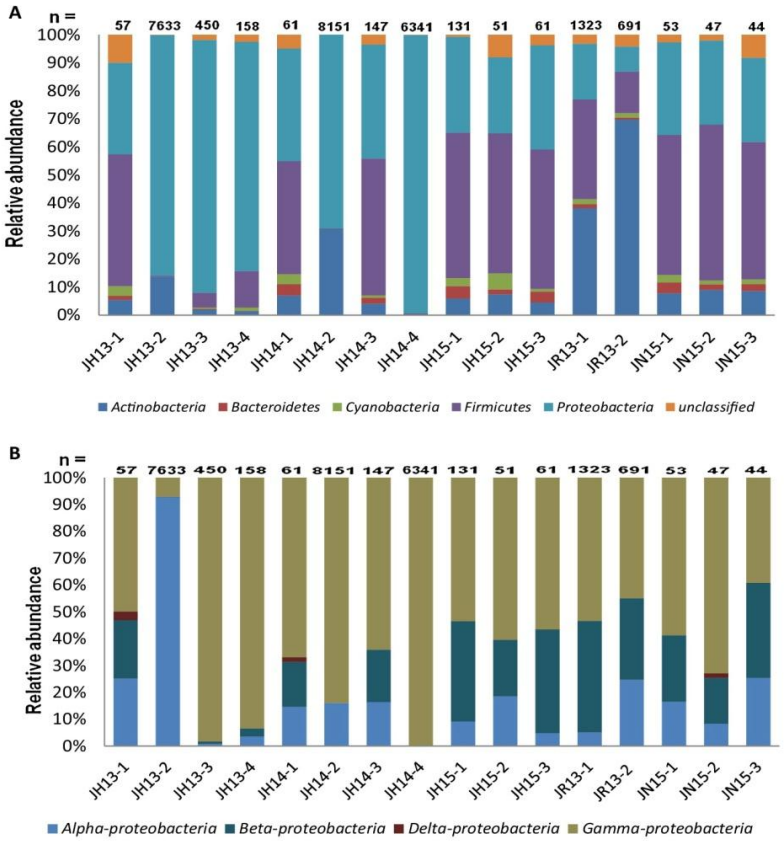

Fig. 5 Dominant phyla of relative abundance of >5% of the reads (**a**) and class distribution of *Proteobacteria* (**b**) detected in in vitro shoot cultures of 16 *Juglans* genotypes

dominated by *Gammaproteobacteria* and genotype JH13-2 being almost exclusively colonized by *Alphaproteobacteria* (Fig. 5b).

Also on genus level, the results of this study clearly showed that the most dominant groups were assigned to the phylum *Proteobacteria*. However, the proportion of *Proteobacteria* was different in the 16 *Juglans* genotypes (Table 4; Supplement 4).

Within the *Alphaproteobacteria, Brevundimonas* spp. showed the highest relative abundance in JH13-2 and JH14-2 (79 and 11%, respectively) and had been isolated from JH13-2 as well. Based on this result and the color and shape of the bacterial colonies, the second isolate from JH14-2 which could not be identified can be assumed to be also a *Brevundimonas* spp. The second OTU with the highest similarity to *Roseomonas* spp. was detected in genotype JH14-3 by Illumina sequencing and also among the isolates. A low relative abundance of *Sphingomonas* spp. was detected in JH14-1, JH15-1 and JN15-3 but had not been isolated from any sample (Table 4).

Betaproteobacteria, which could not be identified in the culture-dependent approach, were observed with six OTUs in the *Juglans* genotypes, one of which was *Burkholderia* spp., which was detected in JH13-4, JH14-3, JH15-1, JH15-2, and JH15-3. The other OTUs had a high similarity to *Ralstonia* spp., *Delftia* spp., *Pelomonas* spp., *Neisseria* spp., and *Azospira* spp. All *Betaproteobacteria* showed a low relative abundance (Table 4).

In the third *Proteobacteria* group, the *Gammaproteobacteria*, an OTU with high similarity to *Acinetobacter* spp. was detected in 13 *Juglans* genotypes. *Acinetobacter* spp. had the highest relative abundance in JH14-2 (58%) and JH14-4 (99%) and it had been also identified by culturing from three genotypes with low propagation rates (JH13-2, JH14-2 and JH14-4). The second most abundant OTU presented the highest similarity to *Proteus* spp., which were detected for all genotypes except JH13-2, JH14-2 and JH14-4. The third one, which was detected both by Illumina MiSeq sequencing and isolation in JH13-3 and JH13-4, had highest similarity to *Moraxella* spp. Finally, only JH14-3 contained *Pseudomonas* spp. and *Erwinia* spp. Dominant genera of other phyla were *Leifsonia* spp. and *Clostridium_sensu_stricto_7* spp.

Table 4 Relative abundance of dominant genera (>5%) detected in 11 *Juglans* hybrid genotypes, 2 *J. regia* genotypes and 3 *J. nigra* genotypes

Phy.*	Bacteria n	JH13-1 57	JH13-2 7633	JH13-3 450	JH13-4 158	JH14-1 61	JH14-2 8151	JH14-3 147	JH14-4 6341	JH15-1 131	JH15-2 51	JH15-3 61	JR13-1 1323	JR13-2 691	JN15-1 53	JN15-2 47	JN15-3 44
1	Actinomyces	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1 ± 2a	0 ± 0a	0 ± 0a	0 ± 0a	2 ± 3a	0 ± 0a	1 ± 1a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
	Corynebacterium	2 ± 4a	0 ± 0	0 ± 0	0 ± 0	2 ± 2a	0 ± 0	1 ± 1a	0 ± 0	0 ± 0	0 ± 0	1 ± 1a	2 ± 3a	0 ± 0	1 ± 2a	4 ± 6a	0 ± 0
	Leifsonia	0 ± 0a	**13 ± 8a**	0 ± 0a	0 ± 0a	0 ± 0a	**30 ± 9b**	0 ± 0a	0 ± 0a	0 ± 0a	1 ± 2a	1 ± 1a	**33 ± 55b**	**64 ± 55b**	1 ± 2a	0 ± 0a	0 ± 0a
	Propionibacterium	0 ± 0a	0 ± 0a	1 ± 0b	1 ± 1a	3 ± 3a	0 ± 0a	1 ± 1a	0 ± 0a	3 ± 2	5 ± 8a	2 ± 2a	3 ± 5a	4 ± 7a	4 ± 1a	5 ± 6a	7 ± 2a
2	Hydrotalea	1 ± 2a	0 ± 0a	0 ± 0a	0 ± 0a	3 ± 6a	0 ± 0a	2 ± 2a	0 ± 0a	3 ± 3a	2 ± 2a	4 ± 2a	1 ± 1a	0 ± 0a	4 ± 0a	2 ± 2a	2 ± 4a
3	Unclassified	2 ± 3a	0 ± 0a	0 ± 0a	1 ± 0a	4 ± 3a	0 ± 0a	1 ± 0a	0 ± 0a	3 ± 4a	6 ± 7a	1 ± 1a	2 ± 3a	2 ± 3a	3 ± 5a	2 ± 1a	2 ± 2a
4	Anoxybacillus	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1 ± 2a	2 ± 3a	0 ± 0a
	Staphylococcus	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1 ± 1a	1 ± 1a	0 ± 0a	0 ± 0a	0 ± 0a	1 ± 2a	1 ± 1a	0 ± 0a
	Clostridium_sensu_stricto_16	2 ± 2a	0 ± 0a	0 ± 0a	1 ± 1a	2 ± 0a	0 ± 0a	3 ± 2a	0 ± 0a	2 ± 2a	7 ± 4a	1 ± 1a	1 ± 2a	2 ± 4a	2 ± 2a	2 ± 2a	1 ± 2a
	Clostridium_sensu_stricto_3	0 ± 0a	0 ± 0a	1 ± 1a	1 ± 1a	1 ± 1a	0 ± 0a	3 ± 1a	0 ± 0a	2 ± 2a	3 ± 1a	3 ± 1a	2 ± 3a	1 ± 2a	2 ± 2a	7 ± 4a	1 ± 2a
	Clostridium_sensu_stricto_7	**28 ± 25b**	0 ± 0a	4 ± 0a	**10 ± 6a**	**32 ± 7c**	0 ± 0a	**40 ± 6c**	0 ± 0a	**42 ± 5c**	**31 ± 7c**	**39 ± 8c**	**32 ± 29c**	**11 ± 17ac**	**40 ± 7c**	**40 ± 6c**	**39 ± 3c**
5.1	Brevundimonas	1 ± 1a	*79 ± 14b*	0 ± 0a	1 ± 1a	2 ± 1a	*11 ± 1c*	1 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
5.1	Roseomonas	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	*3 ± 2b*	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
5.1	Sphingomonas	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1 ± 1a	0 ± 0a	0 ± 0a	0 ± 0a	1 ± 2a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	2 ± 4a
5.2	Burkholderia	0 ± 0a	0 ± 0a	0 ± 0a	1 ± 1a	0 ± 0a	0 ± 0a	2 ± 3a	0 ± 0a	2 ± 3a	1 ± 1a	1 ± 1a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
5.2	Ralstonia	2 ± 3a	0 ± 0a	0 ± 0a	0 ± 0a	1 ± 3a	0 ± 0a	1 ± 2a	0 ± 0a	1 ± 1a	0 ± 0a	1 ± 2a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1 ± 2a
5.2	Delftia	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2 ± 3a	0 ± 0
5.2	Pelomonas	1 ± 2a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	2 ± 2a	0 ± 0a	6 ± 4b	1 ± 2a	2 ± 3a	2 ± 2a	1 ± 2a	2 ± 2a	2 ± 1a	2 ± 4a
5.2	Neisseria	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	2 ± 3a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3 ± 3a
5.2	Azospira	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1 ± 1a	0 ± 0	0 ± 0a	0 ± 0a	2 ± 4a	0 ± 0a	0 ± 0a
5.3	Citrobacter	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3 ± 5a	0 ± 0	1 ± 1a	0 ± 0	0 ± 0	0 ± 0	1 ± 2a	1 ± 2a	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5.3	Erwinia	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4 ± 3a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5.3	Proteus	5 ± 6ab	0 ± 0a	1 ± 1a	3 ± 1a	**10 ± 2b**	0 ± 0a	8 ± 3b	0 ± 0a	**11 ± 6b**	**11 ± 1b**	**10 ± 5b**	7 ± 6ab	4 ± 6ab	9 ± 3b	8 ± 3b	8 ± 8ab
5.3	Acinetobacter	2 ± 2a	5 ± 7a	1 ± 1a	1 ± 1a	2 ± 1a	*58 ± 8b*	1 ± 1a	*99 ± 0b*	1 ± 1a	2 ± 2a	3 ± 1a	0 ± 0a	0 ± 0a	0 ± 0a	6 ± 5a	1 ± 2a
5.3	Moraxella	1 ± 1a	1 ± 1a	*87 ± 3b*	*69 ± 8b*	0 ± 0a	0 ± 0a	1 ± 1a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
5.3	Pseudomonas	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	*4 ± 3b*	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
5.3	Unclassified	2 ± 2	0 ± 0a	0 ± 0a	1.8 ± 1	8.8 ± 5	0 ± 0a	4.6 ± 1	0 ± 0a	5.7 ± 2	4.9 ± 4	8.4 ± 2	4.1 ± 4	1.7 ± 3	7.2 ± 1	8.8 ± 2	2.8 ± 5
6	Unclassified	9 ± 6c	0 ± 0a	2 ± 2ab	3 ± 2ab	5 ± 4bc	0 ± 0a	4 ± 3bc	0 ± 0a	1 ± 1a	8 ± 5bc	4 ± 2b	3 ± 5ab	4 ± 7ab	3 ± 5ab	2 ± 3ab	8 ± 4c

Evaluation by nonparametric ANOVA (Kruskal–Wallis test). Values marked by different letters within the same row are significantly different according to $p < 0.05$. Data are expressed as mean ± SD. Cells in italics indicate bacterial genera that were isolated from in vitro shoots of the respective genotype. Bold marked cells show mean relative abundances >10%. A full list of all OTUs and their relative abundances is offered as Supplement S4

*1, Actinobacteria; 2, Bacteroidetes; 3, Cyanobacteria; 4, Firmicutes; 5, Alphaproteobacteria; 5.2, Betaproteobacteria; 5.3, Gammaproteobacteria; 6, unclassified

Comparing the relative abundance of endophytic bacteria for the same *Juglans* genotype (JH13-3) in samples collected in two different years (July 2015 and July 2016), the composition of the bacterial community had changed. Some OTUs, assigned to *Corynebacterium, Propionibacterium, Streptococcus, Clostridium_sensu_stricto_16 and Brevundimonas*, disappeared, others decreased (*Clostridium_sensu_stricto_7*). Additionally, one OTU assigned to the genus *Moraxella* was observed for the first time in 2016 (Supplement S5).

Discussion

In vitro propagation of a number of *Juglans*×*intermedia* hybrids for planting in forestry has been successfully achieved following the protocol published earlier (Tuan et al. 2016) using embryonic tissue as starting material. Average propagation rates over five subcultures varied between 2.5 and 5.4 (Fig. 1) depending on the genotype with relatively high fluctuation between the culture passages. A similar fluctuation over time was reported for two mature *Juglans* hybrids (*Juglans nigra*×*J. regia*) (Meier-Dinkel and Wenzlitschke 2017) and genotypic differences have been observed in all tissue culture protocols. Nowadays however, the extended phenotype not only is considered to base on its genetic make-up, but also is seen as the result of the plant microbiome which influences plant health and growth (Turner et al. 2013). More recently, also for in vitro cultivated plants, the role of endophytic microorganisms has been studied and was shown to be genotype-dependent and varying over time (Quambusch et al. 2016).

The results of culture-dependent and culture-independent methods applied in the present study indicated that a wide range of microorganisms inhabits the embryonic axes of *Juglans*. Although the bacteria were not observed in most genotypes during the routine micropropagation of *Juglans* shoot cultures derived from the embryonic axes, the presence of bacteria was shown by culturing on bacterial growth media (Figs. 2, 3; Table 2) and by Illumina sequencing (Fig. 4; Table 4; Supplement 4). Presence of bacteria in micropropagation without any symptoms of contamination is not unusual (Leifert et al. 1994; Cassells 1997; Laukkanen et al. 2000). Although the origin of the detected bacteria is not known, it seems that the bacteria derived from the original plant tissue, because not only the embryonic axes from *Juglans* nuts were always carefully sterilized but the explants were also prepared aseptically in all micropropagation procedures. Additionally, the composition of bacteria detected in different *Juglans* genotypes was differing between genotypes. A contamination by human handling, on the other hand, would most likely introduce one strain to many genotypes during the transfer of plantlets. The presence of endophytic bacteria in meristems, flowers and seeds has been shown in other studies, e.g. for *Methylobacterium extorquens* in Scots pine (Koskimäki et al. 2015) and for *Methylobacterium* and *Pantoea* species frequently being detected in seeds of diverse plant species (Mano and Morisaki 2008; Johnston-Monje and Raizada 2011). Endophytic *Pseudomonas* were also identified in meristematic buds of Scots pine (*P. sylvestris*) (Pirttilä et al. 2004) and in Norway spruce seeds (*P. abies*) (Cankar et al. 2005).

All endophytic bacteria isolated from in vitro shoots (culture dependent method) of 16 *Juglans* genotypes were also detected by Illumina MiSeq sequencing (culture-independent method) indicating the reliability of the sequencing data. The isolated strains of the genera *Acinetobacter, Moraxella, Brevundimonas, Pseudomonas* and *Roseomonas* were all detected as OTUs in the same genotype from which they were isolated. The huge differences in sequence reads among the samples were due to very high percentages of chloroplastic and mitochondrial sequences in the samples with low OTU numbers. We assume that in samples with low bacterial titers the primers non-specifically amplified the organelles 16S rRNA fragments. Future studies should employ quantitative PCR analyses to investigate bacterial concentrations in the plant tissues. In samples with very low bacterial DNA content the use of blocking primers could, in addition to the selective primer 799f used in this study, further reduce the chloroplastic and mithochondrial sequences to reach a higher coverage of the bacterial sequences. Arenz et al. (2015) reached a 300-fold increase of bacterial reads with blocking primers compared to standard PCR in an Illumina-based study of *Sorghastrum nutans* leaf endophytes. One previous study investigating epi- and endophytic microorganisms in walnut trees came to the conclusion, that microorganisms in *Juglans* were dominated by fungal taxa, and only 3.6% of all isolated endophytes were bacteria (Pardatscher and Schweigkofler 2009). This might point to a close interaction of bacterial and fungal endophytes under ex vitro conditions which might be shifted under in vitro conditions.

The endophytic bacteria with the highest relative abundance belonged to the phylum *Proteobacteria* such as *Brevundimonas* spp., *Acinetobacter* spp. or *Moraxella* spp. (Fig. 2; Table 4; Supplement 4). Based on the propagation rate, among five *Juglans* hybrid genotypes with high propagation rates, two genotypes (JH13-3 and JH13-4) presented a high relative abundance of the OTU *Moraxella* spp. (Illumina MiSeq sequencing) corresponding to the strain *M. osloensis* (obtained in the culture-dependent approach, Table 4).

Because of rarity, *M. osloensis* has not been well understood yet and it has only been reported as an endophytic bacterium in Durian (*Durio zibethinus* Murr.) (Suhandono

and Utari 2014). Our study showed a clear difference in the relative abundance between two harvesting times for NGS analysis in case of genotype JH13-3 (Supplement S5). One of the reasons for these differences may be a new introduction of the bacteria detected in the tissue culture material (the JH13-3 genotype tested had been established and sub-cultured from 2013). A second explanation could be an increase in the relative abundance of *Moraxella* spp. in high propagation rate genotypes due to the plummeting of other bacteria in JH13-3. A strong fluctuation of the bacterial community in consecutive years of in vitro culture has previously been shown for *P. avium* (Quambusch et al. 2016). The presence in genotypes with high propagation rates could point to a positive influence or mutual relationship of *Moraxella* spp. on *Juglans* shoot cultures in vitro. Further analysis with focus on the interaction as well as a biochemical characterization of the strain would be needed to clarify this.

The strains with closest matches to *B. vesicularis*, and *A. calcoaceticus* were detected in genotypes with lower propagation rates. *Acinetobacter* spp. are gram-negative and, according to Rossau et al. (1991), should be classified into the new family *Moraxellaceae* that includes *Acinetobacter, Moraxella*, and *Psychrobacter. Acinetobacter* spp. were more frequently isolated from forest trees than from agricultural crops (Towner 2006). *Ex-vitro A. calcoaceticus* was observed in stems and rhizospheres of Scots pine (*Pinus sylvestris* L.), silver birch (*Betula pendula* Roth), and rowan (*Sorbus aucuparia* L.) (Izumi et al. 2008), and in stems of native poplar (*Populus trichocarpa*) and willow (*Salix sitchensis*) (Doty et al. 2009). In micropropagation, *Acinetobacter* spp. have been isolated from tissue cultures of *P. avium* L. (Quambusch et al. 2014) and several bacterial strains belonging to *Acinetobacter* have been demonstrated as plant growth promoting bacteria. For instance, *Acinetobacter johnsonii*, isolated from sugar beet roots, was enhancing the growth of sugar beet plants (Shi et al. 2011; Raweekul et al. 2016). Furthermore, *Acinetobacter* spp. could play an important role in bioremediation of numerous hazardous substances (Towner 2006). However, in micropropagation, the *Acinetobacter* spp. were often considered to be a contamination due to their high temperature stability (Leifert et al. 1991; Isenegger et al. 2003; Donnarumma et al. 2011). To our knowledge the species *A. calcoaceticus*, isolated in this study, has not been reported as a plant associated bacterium before.

Brevundimonas vesicularis was previously known under the name *Corynebacterium vesiculare* or *Pseudomonas vesicularis* (Oberhelman et al. 1994) and has been isolated from the seminal vesicle of a leech (Oberhelman et al. 1994; Chi et al. 2004). Additionally, *B. vesicularis* was reported as an endophyte of peach and pear in in vitro rootstock cultures (Liaqat and Eltem 2016) and has been detected in the seed of cotton and cucumber (Hallmann et al. 1998). In contrast, according to Loreti et al. (2009) a *Pseudomonas* spp. was reported to have a negative effect in hazelnut shrubs.

Members of the genera *Pseudomonas, Erwinia* and *Burkholderia*, detected by Illumina sequencing in different *Juglans* genotypes (Table 4), are known as plant growth promoting bacteria (Izumi et al. 2008; Pirttilä 2011). For example, an endophytic strain of *Pseudomonas stutzeri* detected in *Echinacea* tissue culture and *Pseudomonas putida* str. W619 isolated from poplar have been identified as IAA-producing bacteria (Lata et al. 2006; Taghavi et al. 2009). Furthermore, Kamoun et al. (1997) identified *Pseudomonas syringae* strains in microcuttings of *P. cerasus* which could improve the growth in plant tissue culture. Bacteria belonging to the genera *Burkholderia* and *Sphingomonas* were also isolated from stems of poplar (*P. trichocarpa*) and willow (*S. sitchensis*) by Doty et al. (2009).

In conclusion, the present study, using culture-dependent (isolation of bacteria from tissue culture) and culture-independent (Illumina sequencing) methods, for the first time introduced a detailed picture of the bacterial community structure of in vitro shoot cultures of sixteen *Juglans* genotypes including *J. regia, J. nigra* and *Juglans* hybrids on phylum and genus level. A total of eight endophytic bacteria were isolated from the *Juglans* genotypes and identified by sequencing of the 16S rRNA and comparison to the NCBI database. There was a strong overlap between the bacterial communities detected by NGS and by isolation and most of the dominant strains could be cultivated on bacterial growth media. High relative abundances of *Moraxella* spp., *Brevundimonas* spp., *Acinetobacter* spp. in the tissue samples make the isolated strains of these bacteria interesting candidates for further analysis, for instance in inoculation experiments, of their positive or negative influence on propagation and rooting of *Juglans* in vitro. Meanwhile, the abundance of *Moraxella* spp. was correlated with a good micropropagation success in the *Juglans* genotypes under investigation, whereas, *Brevundimonas* spp. and *Acinetobacter* spp. were only detected in clones with medium propagation rates. Future studies should include genotypes with more contrasting propagation rates, as well as quantification of these three bacteria by quantitative PCR or inoculation experiments to investigate their effects on the propagation rate.

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Compliance with ethical standards

Conflict of interest None declared.

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Electronic Supplementary Material (ESM)

Online Resource 1 (ESM1)

Endophytic bacterial communities in *in vitro* shoot cultures derived from embryonic tissue of hybrid walnut (*Juglans* x *intermedia*)

Plant Cell, Tissue and Organ Culture (PCTOC) - Journal of Plant Biotechnology

Pham Ngoc Tuan[1,2,3], Andreas Meier-Dinkel[1*], Aki M. Höltken[1], Mona Quambusch[1], Felix Mahnkopp[2] , and Traud Winkelmann[2].

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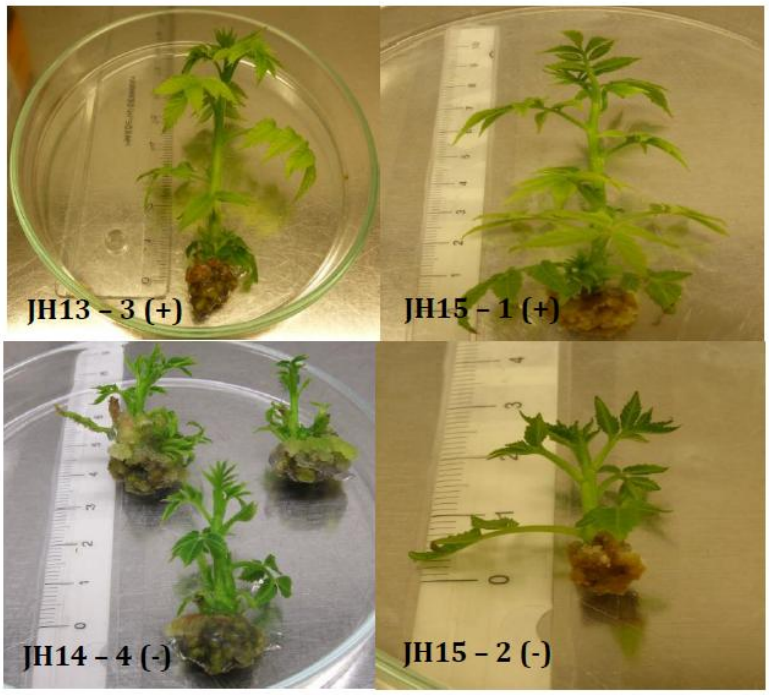
ESM1: Appearance of shoot clusters at the end of a culture passage: *Juglans* hybrid genotypes with good (+) and medium (-) propagation rates: JH13-3 (+), JH15-1 (+), JH14-4 (-) and JH15-2 (-)



Electronic Supplementary Material (ESM)

Online Resource 2 (ESM2)

Endophytic bacterial communities in *in vitro* shoot cultures derived from embryonic tissue of hybrid walnut (*Juglans* x *intermedia*)

Plant Cell, Tissue and Organ Culture (PCTOC) - Journal of Plant Biotechnology

Pham Ngoc Tuan[1,2,3], Andreas Meier-Dinkel[1*], Aki M. Höltken[1], Mona Quambusch[1], Felix Mahnkopp[2] , and Traud Winkelmann[2].

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ESM2: The count tables for the OTUs for *in vitro* shoot cultures of 16 *Juglans* genotypes using Illumina MiSeq sequencing technology.

Nr.	Genotypes	Number of. OTUs
1	JH13-1	15
2	JH13-1	19
3	JH13-1	21
4	JH13-3 (II)*	29
5	JH13-3 (II)*	22
6	JH13-3 (II)*	11
7	JH14-1	18
8	JH14-1	20
9	JH14-1	27
10	JH13-2	21
11	JH13-2	33
12	JH13-2	27
13	JH14-2	29
14	JH14-2	16
15	JH14-2	18
16	JH13-4	23
17	JH13-4	18
18	JH13-4	15
19	JH14-3	27
20	JH14-3	32
21	JH14-3	37
22	JH14-4	15
23	JH14-4	23
24	JH14-4	31

25	JH15-1	40
26	JH15-1	10
27	JH15-1	30
28	JH15-2	19
29	JH15-2	24
30	JH15-2	13
31	JH15-3	15
32	JH15-3	24
33	JH15-3	21
34	JR13-1	13
35	JR13-1	17
36	JR13-1	27
37	JR13-2	17
38	JR13-2	11
39	JR13-2	16
40	JN15-1	25
41	JN15-1	14
42	JN15-1	15
43	JN15-2	21
44	JN15-2	7
45	JN15-2	22
46	JN15-3	14
47	JN15-3	21
48	JN15-3	12
	Total	995
49	JH13-3(I)*	29
50	JH13-3(I)*	22
51	JH13-3(I)*	19
	Total	1,065

*Genotype JH 13-3 was collected in two consecutive years for analysis.

Electronic Supplementary Material (ESM)

Online Resource 3 (ESM3)

Endophytic bacterial communities in *in vitro* shoot cultures derived from embryonic tissue of hybrid walnut (*Juglans* x *intermedia*)

Plant Cell, Tissue and Organ Culture (PCTOC) - Journal of Plant Biotechnology

Pham Ngoc Tuan[1,2,3], Andreas Meier-Dinkel[1*], Aki M. Höltken[1], Mona Quambusch[1], Felix Mahnkopp[2] , and Traud Winkelmann[2].

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ESM 3 Relative abundance of dominant phyla detected in 16 *Juglans* hybrids and species

Phyla n	JH13-1 57	JH13 - 2 7633	JH13 - 3 450	JH13 -4 158	JH14 - 1 61	JH14 - 2 8151	JH14 - 3 147	JH14 - 4 6341	JH15 - 1 131	JH15 -2 51	JH15 -3 61	JR13 -1 1323	JR13 -2 691	JN15 -1 53	JN15 - 2 47	JN15 -3 44
Acidobacteria	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.1±0 .2a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Actinobacteria	5.4±8.1a	13.7± 7.7a	2.0±0 .9a	1.4±0 .7a	7.0±4 .5a	30.6± 9.2b	4.0±0 .6a	0 ± 0 a	5.9±5 .7a	7.4±8 .6a	4.5±3 .9a	38.2± 52.7b	69.7±48 .2b	7.7±3 .4a	8.9±1 1.5a	8.5±3 .9a
Bacteroidetes	1.4±2.5a	0 ± 0a	0.3±0 .3a	0.1±0 .2a	4.0±5 .7a	0.0a	2.2±2 .2a	0.0 ± 0.1a	4.3±3 .1a	1.7±1 .8a	3.9±2 .1a	1.4±1 .3a	0.6±0.8a	3.8±0 .2a	2.0±1 .8a	2.4±4 .1a
Candidate_division_TM7	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.9±1 .6a	0 ± 0	0 ± 0
Chloroflexi	1.0±1.7a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Cyanobacteria	3.4±2.1a	0 ± 0	0.4±0 .2a	1.2±0 .3a	3,.6± 3.4a	0 ± 0	0.8±0 .4a	0 ± 0	2.9±3 .7a	5.7±7 .0a	0.9±1 .5a	1.8±3 .1a	1.7±2.6a	2.7±4 .6a	1.5±1 .5a	1.7±1 .8a
Deinococcus-Thermus	0 ± 0a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0a	0.4±0 .7a	0 ± 0a	0.6±1 .0a	0.5±0 .9a	0 ± 0 a	0 ± 0a	0 ± 0 a	0 ± 0 a	0 ± 0a	1.2±2 .1a
Firmicutes	46.6±4. 7b	0.4±0 .3a	5.3±0 .2a	13.0± 6.3a	40.4± 1.2b	0.2±0 .0a	48.6± 9.3b	0.5±0 .3a	51.4± 7.5b	49.7± 1.3b	49.8± 7.2b	35.6± 31.0b	14.8±2. 7a	49.5± 12.5b	55.6± 10.4b	48.4± 2.8b
Proteobacteria	32.4±1. 3a	85.8± 7.7b	90.2± 2.5b	81.9± 6.2b	40.2± 1.8a	69.1± 9.1b	40.4± 9.8a	99.3± 0.3b	33.9± 9.2a	27.0± 8.7a	37.2± 6.6a	19.9± 16.6a	8.9±13. 9a	32.7± 7.5a	29.9± 2.9a	29.7± 4.1a
Spirochaetae	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.2±0 .3a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
unclassified	9.9±3.6 b	0.1±0 .0a	1.8±1 .6a	2.5±1 .7a	4.9±3 .7b	0 ± 0a	3.5±3 .2a	0.1±0 .1a	0.7±0 .9a	8.0±5 .0a	3.7±1 .7a	3.2±5 .4a	4.2±7.2a	2.7±4 .6a	2.0±3 .4a	8.1±4 .0a

Evaluation by nonparametric ANOVA (Kruskal–Wallis testing). Values followed by the same letter in each row are not significantly different according to $p<0.05$. Data are expressed as mean ± SD.

Electronic Supplementary Material (ESM)

Online Resource 4 (ESM4)

Endophytic bacterial communities in *in vitro* shoot cultures derived from embryonic tissue of hybrid walnut (*Juglans* x *intermedia*)

Plant Cell, Tissue and Organ Culture (PCTOC) - Journal of Plant Biotechnology

Pham Ngoc Tuan[1,2,3], Andreas Meier-Dinkel[1*], Aki M. Höltken[1], Mona Quambusch[1], Felix Mahnkopp[2] , and Traud Winkelmann[2].

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ESM 4 Relative abundance of dominant genera (>1%) detected in 11 *Juglans* hybrid genotypes, 2 *J. regia* genotype and 3 *J. nigra* genotypes

Phy. *	Bacteria n	JH13-1 57	JH13- 2 7633	JH13- 3 450	JH13-4 158	JH14- 1 61	JH14- 2 8151	JH14- 3 147	JH14- 4 6341	JH15- 1 131	JH15-2 51	JH15-3 61	JR13-1 1323	JR13-2 691	JN15-1 53	JN15- 2 47	JN15-3 44
1	*Actinobaculum*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±2a
	Actinomyces	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±2a	0 ± 0a	0 ± 0a	0 ± 0a	2±3a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
	Corynebacterium	2±4a	0 ± 0	0 ± 0	0 ± 0	2±2a	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0	1±1a	2±3a	0 ± 0	1±2a	4±6a	0 ± 0
	Curtobacterium	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±0b	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±2ab	0 ± 0a	0 ± 0a
	Leifsonia	0 ± 0a	13±8a	0 ± 0 a	0 ± 0a	0 ± 0a	30±9b	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±2a	1±1a	33±55 b	64±55 b	1±2a	0 ± 0 a	0 ± 0 a
	Leucobacter	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0
	unclassified	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±0a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0
	Rothia	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Microlunatus	1±3a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Propionibacterium	0 ± 0a	0 ± 0a	1±0b	1±1a	3±3a	0 ± 0	1±1a	0 ± 0 a	3±2	5±8a	2±2a	3±5a	4±7a	4±1a	5±6a	7±2a
2	*Prevotella*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Cecembia	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0
	Hydrotalea	1±2a	0 ± 0a	0 ± 0a	0 ± 0a	3±6a	0 ± 0a	2±2a	0 ± 0a	3±3a	2±2a	4±2a	1±1a	0 ± 0a	4±0a	2±2a	2±4a
3	unclassified	2±3a	0 ± 0 a	0 ± 0 a	1±0a	4±3a	0 ± 0 a	1±0a	0 ± 0 a	3±4a	6±7a	1±1a	2±3a	2±3a	3±5a	2±1a	2±2a
4	*Thermus*	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±1a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±0a
5	*Anoxybacillus*	0 ± 0a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±2a	2±3a	0 ± 0a
	Exiguobacterium	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±1a	0 ± 0 a
	Staphylococcus	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±1a	1±1a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±2a	1±1a	0 ± 0 a

	Enterococcus	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±1a	1±2a	1±1a	0 ± 0 a	0 ± 0 a	1±2a	0 ± 0a	1±2a
	Lactobacillus	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	2±2a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±3a	0 ± 0 a
	Streptococcus	0 ± 0 a	0 ± 0 a	0 ± 0a	1±1a	0 ± 0	0 ± 0 a	1±1a	0 ± 0 a	2±2a	0 ± 0 a	1±1a	0 ± 0 a	0 ± 0 a	2±2a	0 ± 0a	2±2a
	Clostridium_sensu_stricto_16	2±2a	0 ± 0 a	0 ± 0a	1±1a	2±0a	0 ± 0 a	3±2a	0 ± 0 a	2±2a	7±4a	1±1a	1±2a	2±4a	2±2a	2±2a	1±2a
	Clostridium_sensu_stricto_3	0 ± 0 a	0 ± 0 a	1±1a	1±1a	1±1a	0 ± 0 a	3±1a	0 ± 0 a	2±2a	3±1a	3±1a	2±3a	1±2a	2±2a	7±4a	1±2a
	Clostridium_sensu_stricto_7	28±25 b	0 ± 0a	4±0a	10±6a	32±7c	0 ± 0a	40±6c	0 ± 0a	42±5c	31±7c	39±8c	32±29 c	11±17 ac	40±7c	40±6c	39±3c
	Anaerococcus	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±1a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	Finegoldia	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±1a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±1a	0 ± 0 a
	Peptoniphilus	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±1a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	Blautia	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0	0 ± 0 a	0 ± 0 a	0 ± 0 a	1±1a	0 ± 0 a
	unclassified	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	4±5a	0 ± 0a	0 ± 0a	0 ± 0a	2±2a	3±1a	3±1a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	3±4a
6	*Brevundimonas*	1±1a	79±14 b	0 ± 0a	1±1a	2±1a	11±1c	1±0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
	Caulobacter	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±2a	0 ± 0a	3±1a
	Bradyrhizobium	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	0 ± 0a	3±2a	0 ± 0a	0 ± 0a
	Methylobacterium	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	2±2a	0 ± 0a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	0 ± 0a
	Mesorhizobium	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	1±2a
	Roseomonas	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	3±2b	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
	Candidatus_Alysiosphaera	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Reyranella	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	0 ± 0a	1±1a	0 ± 0a
	Novosphingobium	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Sphingomonas	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	0 ± 0a	1±2a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	2±4a
	Alcaligenes	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Burkholderia	0 ± 0a	0 ± 0a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	2±3a	0 ± 0a	2±3a	1±1a	1±1a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
	Ralstonia	2±3a	0 ± 0a	0 ± 0a	0 ± 0a	1±3a	0 ± 0a	1±2a	0 ± 0a	1±1a	0 ± 0a	1±2a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±2a
	Curvibacter	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Delftia	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2±3a	0 ± 0
	Pelomonas	1±2a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	2±2a	0 ± 0a	6±4b	1±2a	2±3a	2±2a	1±2a	2±2a	2±1a	2±4a
	Aquitalea	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±1a	1±1a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0
	Neisseria	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	2±3a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3±3a
	Azospira	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±1a	0 ± 0	0 ± 0a	0 ± 0a	2±4a	0 ± 0a	0 ± 0a
	Syntrophus	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Aeromonas	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0
	Succinivibrio	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±2a	0 ± 0	0 ± 0
	Cedecea	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±2a	0 ± 0a	1±2a	0 ± 0a	1±1a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	1±2a	0 ± 0a	0 ± 0a
	Citrobacter	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3±5a	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0	1±2a	1±2a	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Erwinia	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	4±3a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Klebsiella	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2±1a	0 ± 0	0 ± 0	0 ± 0	1±1a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±2a	0 ± 0	0 ± 0

	Morganella	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±2a	0 ± 0	0 ± 0
	Proteus	5±6ab	0 ± 0a	1±1a	3±1a	10±2b	0 ± 0a	8±3b	0 ± 0a	11±6b	11±1b	10±5b	7±6ab	4±6ab	9±3b	8±3b	8±8ab
	Haemophilus	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±2a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1±2a	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	Acinetobacter	2±2a	5±7a	1±1a	1±1a	2±1a	58±8b	1±1a	99±0b	1±1a	2±2a	3±1a	0 ± 0a	0 ± 0a	0 ± 0a	6±5a	1±2a
	Enhydrobacter	1±1a	1±1a	87±3b	69±8b	0 ± 0a	0 ± 0a	1±1a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
	Psychrobacter	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	1±1a	1±1a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
	Pseudomonas	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	4±3b	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a	0 ± 0a
	unclassified	5±4a	1±0a	1±0a	3±1a	11±4b	0 ± 0a	8±2b	0 ± 0a	7±3b	8±7b	12±4b	8±7ab	3±4a	8±1b	10±1b	5±8a
7	unclassified	9±6c	0 ± 0a	2±2ab	3±2ab	5±4bc	0 ± 0a	4±3bc	0 ± 0a	1±1a	8±5bc	4±2b	3±5ab	4±7ab	3±5ab	2±3ab	8±4c

*Relative abundance of >1% total sequences in the sample were selected. Evaluation by nonparametric ANOVA (Kruskal–Wallis testing). Values in a row followed by the same letter are not significantly different according to $p<0.05$. Data are expressed as mean ± SD, * Phyla: 1:* Actinobacteria, *2:* Bacteroidetes, *3:* Cyanobacteria, *4:* Deinococcus-Thermus 5: Firmicutes, *6:* Proteobacteria, *7: unclassified.*

Electronic Supplementary Material (ESM)

Online Resource 5 (ESM5)

Endophytic bacterial communities in *in vitro* shoot cultures derived from embryonic tissue of hybrid walnut (*Juglans* x *intermedia*)

Plant Cell, Tissue and Organ Culture (PCTOC) - Journal of Plant Biotechnology

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ESM 5 Relative abundance of dominant genera (>5%) detected in the *Juglans* genotype JH13-3

Phylum	Bacteria	**JH13 – 3 (I)**	**JH13 – 3 (II)**
Actinobacteria	*Corynebacterium*	2±3	0±0
	Propionibacterium	3±3	1±0
Firmicutes	*Streptococcus*	2±3	0±0
	Clostridium_sensu_stricto_16	3±6	0±0
	Clostridium_sensu_stricto_7	14±12	4±0
Proteobacteria	*Brevundimonas*	39±47	0±0
	Caulobacter	0±0	0±0
	Pelomonas	0±0	0±0
	Acinetobacter	1±1	1±1
	Moraxella	0±0	87±3
	unclassified	3±3	0±0

Relative abundance of >5% total sequences in the sample were selected. Evaluation by nonparametric ANOVA (Kruskal–Wallis testing) according to $p<0.05$. Data are expressed as mean ± SD

Manuscript 2.4

Mating structures in an open-pollinated hybridogenic Black Walnut tree (*Juglans nigra* L.) producing *Juglans* x *intermedia* progeny.

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Type of article:	Research article
Contribution to the article:	Contributed in preparing experiments, data analysis, discussion of results and writing the paper.
Contribution of other authors:	*Aki Höltken*: Planned and performed experiments, analyzed the data, prepared figures and tables, wrote the paper. *Andreas Meier-Dinkel* & *Traud Winkelmann*: contributed in design of experiments, data analysis and writing the paper.
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Mating structures of an open-pollinated hybridogenic Black walnut tree (*Juglans nigra* L.) producing *Juglans* x *intermedia* progeny

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Abstract

Hybrid walnut (*Juglans* x *intermedia* = *J. nigra* ♀ x *J. regia* ♂) has raised interest due to its valuable timber, excellent growth rate and low biotic/abiotic vulnerability. To select elite genotypes, multiple clones have to be tested with a sufficient genetic variability. However, the increasing demand is accompanied by a low availability of reproductive material: (1) hybrid plant production depends on spontaneous natural hybridization of very few `hybridogenic´ trees, (2) artificial pollination is difficult and (3) vegetative propagation needs *in vitro* techniques using embryos or (much more difficult) buds.

This study focuses on the genetic structure of the progeny of a single hybridogenic *J. nigra* tree. It reveals that an already limited number of microsatellite (=SSR) markers gives a precise species differentiation to detect hybrids in early ontogenetic stages (embryo tissue) for micropropagation. Genetic variation and allelic profiles of the successful *J. regia* pollen show that diverse pollen donors have contributed to the genetic composition of hybrid progeny resulting in a broad genetic base for further use in field trials. The genetic structure of *J. nigra* pollen contributing to intraspecific matings disclose a distinctly lower genetic variability compared to reference samples and a high estimated proportion of self-

fertilization (> 20%). Although both species are characterized by dichogamous flowering, the period of female flower receptivity of the hybridogenic *J. nigra* tree seems to overlap with the period of mature pollen release of both, its own male flowers as well as male inflorescences of surrounding *J. regia* and *J. nigra* trees.

Introduction

The demand for noble hardwood timber will continue to rise as the worldwide consumption of forest products increases, and, at the same time, political and environmental pressures are leading to an expansion of protected forest areas. In Germany at least 10% of the state and corporation forests are aimed to be protected until 2020 in the framework of the National Biodiversity Strategy and Action Plan. Limitations or even total prohibition of harvesting will particularly affect (semi-)natural forests with higher proportions of high-quality noble hardwood trees (STÜBNER et al. 2012, WILDMANN et al. 2014). Consequently, silvicultural strategies have to be adapted on the remaining forest area and tree improvement programs for high-quality hardwood tree species to be developed for production forestry (PIJUT et al. 2007).

Forest tree breeders are interested in developing populations with superior growth, timber quality and biotic/abiotic resistance/tolerance. In this regard, *Juglans* x *intermedia*, a hybrid between *Juglans nigra* L. (Eastern black walnut. ♀) and *Juglans regia* L. (Persian walnut. ♂), appeared to be a very interesting alternative for forestry in Central and Northern Europe due to outstanding characteristics concerning timber quality, growth characteristics and adaptability. This interspecific cross is superior to the parents in growth at sites with medium to high fertility and moderately tolerant to flooding (tolerance to anoxia stress for 10 to 12 days). It combines most of the positive features of both parental species such as strong apical dominance, a better winter-hardiness and resistance to spring frost damage than *J. regia*, straightness of boles as well as more `regia´ typic coloration of the timber (see e.g. POLLEGIONI et al. 2010, TUAN et al. 2016).

Although belonging to different sections of the genus *Juglans*, hybrid progeny can occur naturally. However, Black walnut trees that show a particular aptidude for interspecific matings with Persian walnut are scarce due to mechanisms of incompatibility and absence of flowering synchrony. Thus, there are only two hybrid walnut sources available: 1. the progenies of two *J. nigra* mother trees pollinated by two *J. regia* pollen donors (on the market as the French varieties NG23 and NG28) and 2. the progeny of one Black walnut

tree pollinated by an unknown number of Persian walnut fathers under the trade name RENI from South Germany (nursery Anton Schott, Leiselheim, South Germany).

In order to select an improved hybrid walnut planting stock for production forestry (i. e. for plantations), a multiclonal variety of *Juglans* hybrids with an adequate number of genotypes and a sufficient broad genetic base has to be tested on different ecological sites. However, *Juglans* hybrids have a very low genetic and/or physiological capacity for adventitious root formation and appeared to be recalcitrant to routine, commercial-scale vegetative propagation. Therefore, *in vitro* protocols have been established and improved in recent years using embryo axes as source material (see e.g. CORNU 1988, CORNU & JAY-ALLEMAND 1989, JAY-ALLEMAND et al. 1992, CHELAWANT et al. 1995, TUAN et al. 2016).

This study focuses on the above mentioned single hybridogenic tree in the nursery of Anton Schott and part of its F1 offspring of the years 2013, 2014 and 2015 (RENI). The offspring may be either Black walnut if the pollen donor is also a Black walnut (*J. nigra*) or hybrid walnut (*J.* x *intermedia*) if the paternal parent is a Persian walnut (*J. regia*). The proportion of hybrid walnuts is estimated at 20 to 60%, varying according to the year (Anton Schott, pers. comm.). Microsatellite (SSR-) markers are analyzed in order to provide information about the genetic diversity of the progeny as a basis for improvement and field testing programs as well as for early selection and use in propagation of superior genotypes in production (plantation) forestry. Basic population genetic parameters (indices of diversity. heterozygosity) are compared with reference collections of the parental species and the discriminatory power regarding taxonomic species differentiation is described (genetic differentiation).

In recent investigations, the proportion of hybrid progeny was found to be very low compared to the particular hybridogenic tree in this study, even if an ample flowering synchrony was observed between female receptive stigmas of *J. nigra* and pollen releasing catkins of the male flowers of *J. regia* (POLLEGIONI et al. 2010). Therefore, in the present study particular attention is paid on the genetic structures of the pollen, i.e. the male contribution to the progeny giving first insights into the mating system of this single hybridogenic tree. Genetic diversity parameters and allelic profiles of the pollen of presumed pollinators as well as paternity analyses are discussed regarding potential mating preferences.

Materials and Methods

Plant material

Reference material: Leaf samples of clearly morphologically determined adult trees during full foliage in summer (52 Black walnut and 48 Persian walnut trees) were collected from a seed orchard, a forest botanical garden and two nurseries in Germany as a reference data set in order to characterize the genetic diversity and allelic profiles of different SSR-markers for species and hybrid identification.

Hybridogenic mother tree: A sample of the outer green pericarp of a single `hybridogenic´ *J. nigra* tree was taken on the nursery area of Anton Schott, Leiselheim, South Germany

Progeny: 263 nuts and young seedlings were collected from the above mentioned single `hybridogenic´ Black walnut tree over three years.

Potential pollen donors: Leaf samples of two Persian and 21 Black walnut trees, located in the surrounding of the single hybridogenic *J. nigra* tree, were collected as potential male mating partners. Almost all potential *J. nigra* trees were recorded within a radius of 0.1 and 3.5 km. Because *J. regia* is very frequently represented, a full inventory would have overburdened this project financially. Thus, we only sampled leaves of two potential fathers (distance about 0.1 km) flowering simultaneously with the hybridogenic mother tree (according to observations of Anton Schott).

All the plant material analyzed is listed in Table 1.

Table 1: Amount, sampling location and sampling year of genetically analyzed individuals; sampling locations of reference leaf material

Material	Individuals	Sampling location	Sampling year
Juglans nigra reference samples	52	seed orchard NW-FVA[1], forest botanical garden[2]	2013
Juglans regia reference samples	48	seed orchard NW-FVA[1], nursery NW-FVA[3]	2013
hybridogenic *Juglans nigra* tree	1	private nursery[4]	2013
Potential fathers *Juglans nigra*	21	surrounding of hybridogenic *Juglans nigra* tree	2015
Potential fathers *Juglans regia*	2	surrounding of hybridogenic *Juglans nigra* tree	2015
Progeny (nuts, seedlings, already established *in-vitro* material)	263	hybridogenic *Juglans nigra* tree, private nursery[4]	2014/2015

1. Seed orchard with *Juglans regia* and *J. nigra* in Oldendorf (Lower Saxony); 2. Forest Botanical Garden in Hann. Münden (Lower Saxony); 3. nursery of the NW-FVA (Northwest German Forest Research Institute) and 4. private nursery in Southwest Germany (Anton Schott)

SSR-analyses

DNA-extraction: Leaf samples were collected from *in vivo* material (one leaf per adult tree and seedling). *In vitro* material was analyzed using embryo, callus, stem and leaf tissues. One centimetre-squared of a single leaf or about 50 mg of *in vitro* material (wet-weight) were placed in 2 ml Eppendorf cups. After grinding the frozen plant material with a mixer mill (MM 300, Retsch), DNA was extracted using the protocol of (DUMOLIN et al. 1995) based on CTAB/dichlormethane. DNA concentrations were then measured (Nanodrop ND-1000, Peqlab, Erlangen, Germany) and dilutions containing 10 ng/µL DNA were prepared and stored at –20°C.

SSR-Genotyping: Six microsatellites (WGA027, WGA118, WGA089, WGA331, WGA069, WGA276; see Table 3) were amplified according to the PCR protocols in POLLEGIONI et al. (2009a, 2012). PCR amplification fragments were resolved by capillary electrophoresis on a GeXP Genetic Analysis System (Beckman Coulter).

Statistics

Population genetic parameters: Using the GSED software (GILLET 2010), the following parameters were estimated to describe allelic variation: The total and average number of alleles found at each locus and the genetic diversity (= effective number of alleles). According to GREGORIUS (1978), the genetic diversity measures the effective number of genetic types taking into account their frequencies in a population. In the version $v_2 = \left(\sum_{i=1}^{n_k}(p_i)^2\right)^{-1}$, where p_i := frequency of the *i*-th allele (i = 1.2.….n_k), it equals the effective number of alleles (N_e) of CROW and KIMURA (1979. p. 324). Further we calculated the observed and expected heterozygosities (H_o and H_e. respectively). The fixation index $F = 1 - H_o/H_e$ obtains information about homozygote or heterozygote excess compared to Hardy-Weinberg conditions (HEDRICK 2011).

In order to genetically differentiate between the two species *J. regia* and *J. nigra*, the detection of private alleles was performed using the software GENALEX 6 by PEAKALL & SMOUSE (2006). The efficiency of the SSR-Marker for species differentiation was further underlined by calculating a genetic distance parameter (GREGORIUS 1974, GREGORIUS & ROBERDS 1986) measuring the proportion of alleles not shared by both of the species:

$0 \leq d_{NR} = 0.5 \cdot \sum_{i=1}^{n_k} |n_i - r_i| \leq 1$, where the n_i´s and the r_i´s denote the allelic structures of locus *i* in the species *J. nigra* (*N*) and *J. regia* (*R*).

Species differentiation: In order to assign the individuals into taxonomic groups (*Juglans nigra* and hybrids *J.* x *intermedia*), we used the Principle Coordinate Analysis (PCoA) displaying the pairwise (squared) Eucledian distances of the genotypes (SMOUSE & PEAKALL 1999) in a bidimensional plot (GenAlex software package according to PEAKALL & SMOUSE 2006). We further used the software STRUCTURE 2.3.4. In the latter case we abstained from displaying the findings in form of bar charts, because those results were already described in detail in a recent publication (TUAN et al. 2016).

Genetic structure of the pollen contribution in Juglans nigra and J. x intermedia progeny genotypes: The distinction of the male and female gametic haplotypes in each offspring genotype is an important prerequisite for the estimation of the genetic contribution of the pollen to a plant progeny´s genetic structure. In the case of codominantly inherited DNA-markers (here: microsatellites = SSRs), direct counting of alleles contributed by pollen is always possible for single-tree progenies of homozygous plants. For the genotype A_iA_i, the frequency of the allele A_k among the successful pollen is simply the frequency N_{ik} of the genotype A_iA_k among the progeny ($k = 1,\ldots, n$). For single plant progenies of heterozygous plants (genotype A_1A_2), however, determination of the proportion of all pollen contributions is not possible, if the pollen can carry both of the alleles present in the mother tree. In the case of heterozygous mother trees, the allelic contribution of the successful pollen donors can be calculated as follows (see GILLET 1997):

1. Maximum-likelihood estimation of the frequency of the successful pollen alleles p^*_1, $p^*_2,\ldots p^*_n$ for the maternal parent of genotype A_1A_2 and progeny sample with genotype frequencies $N = (N_{11}, N_{22}, N_{12}, N_{1k}, N_{2k})_{k = 3,\ldots n}$ assuming regular segregation of maternal alleles:

$$(p_1^*,\ p_2^*) = \begin{cases} (0,0) & \text{if } N_{11} = N_{22} = N_{12} = 0 \\ \left(\dfrac{N_{11} \cdot q}{N_{11} + N_{22}}, \dfrac{N_{22} \cdot q}{N_{11} + N_{22}}\right) & \text{if } N_{11} + N_{22} > 0 \\ \{(a, q - a) | a \in [0, q]\} & \text{if } N_{11} = N_{22} = 0,\ N_{12} > 0 \end{cases}$$

where $q = (N_{11} + N_{22} + N_{12})/N$.

2. Estimation of the frequency of alleles p_k which are not existent in the mother tree:

$$p_k^* = \left(\frac{N_{1k} + N_{2k}}{N}\right) \qquad (k = 3, \ldots n)$$

Paternity analysis: For the paternity assignment we used the Cervus 3.03 computer program (statistical background: MARSHAL et al. 1998). Allele frequencies were used to perform simulation analyses to establish a confidence threshold for assignment. To infer paternity, LOD scores (log of the ratio of the likelihood given parentage to likelihood given non-parentage) were calculated for each possible paternity. Critical LOD values were derived from simulations involving 50.000 offspring. A 0.5% genotyping error was assumed, the proportion of candidate parents sampled was set to 0.7 and 0.9.

Results

Population genetic structures in reference collections of *J. nigra* and *J. regia*

Clear differences can be observed in the genetic diversity parameters between the two species *J. nigra* and *J. regia* based on collections of adult trees in nurseries as well as planted individuals or stands in German forests. Basic population genetic indices are listed in Table 2.

Table 2: Measures of genetic variability in reference collections of *Juglans regia* and *J. nigra*; name of SSR-Loci, number of observed alleles (N_A), effective number of alleles (N_e), observed and expected heterozygosity (H_o and H_e. respectively), fixation index (F)

	Juglans regia					*Juglans nigra*				
Locus	N_A	N_e	H_o	H_e	F	N_A	N_e	H_o	H_e	F
WGA027	2	1.97	0.33	0.49	0.32	18	6.48	0.73	0.85	0.14
WGA118	4	1.82	0.42	0.42	0.00	16	5.07	0.85	0.80	-0.06
WGA089	3	2.30	0.52	0.56	0.08	16	9.64	0.84	0.89	0.07
WGA331	3	1.75	0.29	0.43	0.32	13	3.34	0.63	0.70	0.10
WGA069	6	2.47	0.40	0.60	0.34	8	2.33	0.67	0.57	-0.18
WGA276	10	3.20	0.73	0.69	-0.06	15	5.74	0.75	0.83	0.09
Mean	4.67	2.16	0.45	0.53	0.17	14.33	4.42	0.74	0.77	0.03

The genetic diversity of the Persian walnut in this reference collection proved to be much lower than that of the Eastern black walnut. The number of alleles found at the six analyzed microsatellite loci ranged from 2 to 10 with a mean of 4.67 for *J. regia* whereas more than three times more alleles (N_A = 14.33) were observed on average in *J. nigra* ranging from 8 to 18 alleles per locus. The same holds true for the effective number of alleles N_e taking into account the frequencies of each allele. The harmonic mean value of this parameter was more than two times higher in *J. nigra* (> 4.4) than in *J. regia* (< 2.2).

Corresponding to the genetic diversity, the expected heterozygosity is much higher in *J. nigra* than in *J. regia* (0.77 and 0.53, respectively) due to the direct relationship to the effective number of alleles ($H_e = 1 - 1/N_e$). Otherwise, the fixation index F was much higher in *J. regia* (0.17) than in *J. nigra* (0.03) due to homozygote excess (=heterozygote deficit) at the SSR-loci WGA027, WGA331 and WGA069 (F = 0.32 to 0.34). The range of the fixation indices over the loci was comparatively high for both species, altogether slightly lower for *J. nigra* (-0.176 to 0.142) than for *J. regia* (-0.060 to 0.335).

Genetic differentiation between taxonomic groups: Pure species and hybrids

Table 3 gives information about genetic differences and the power of the used microsatellites in order to genetically differentiate the two walnut species. Private alleles were detected for both species at each single SSR-locus. Overall, 1 to 6 private alleles have been detected for *J. regia* (mean over all loci: 3 private alleles). That means, almost 2/3 of all allele findings turned out to be species specific based on the collection of Persian walnut individuals. Much higher was the number of private alleles in *J. nigra*. More than 88% of the alleles were observed in Black walnut exclusively (6 to 17 private alleles at each of the analyzed SSR-loci).

Table 3: Parameters of genetic differentiation between the species *Juglans regia* and *J. nigra*; number of private alleles ($N_{private}$), frequency of private alleles ($p_{private}$) for both species and genetic distance d_0 between species

	Juglans regia		***Juglans nigra***		**Genetic distance**
Locus	$N_{private}$	$p_{private}$	$N_{private}$	$p_{private}$	d_0
WGA027	1	0.438	17	0.979	0.984
WGA118	3	0.260	15	0.993	0.995
WGA089	2	0.927	15	0.904	0.927
WGA331	2	0.302	12	0.993	0.935
WGA069	4	0.832	6	0.822	0.897
WGA276	6	0.677	11	0.911	0.962
Mean	3.000	0.573	12.667	0.934	0.950

Also the relative frequencies of the observed private alleles differed strongly between the two species. In *J. regia* the frequency of private alleles ranges from 0.260 and 0.927 (average: 0.573), whereas this value is much higher in *J. nigra* with 0.822 and 0.993 (average: 0.934). The reason for this difference is, that *J. regia* shares several alleles with *J. nigra*, which are present with quite high frequencies in *J. regia* but with very low frequencies in *J. nigra*. This is underlined by the genetic distances between the two species. The parameter d_0 varied between loci from 0.897 and 0.995. The average value is 0.950, that means the two species show a genetic difference of 95.0 % (based on the

analyzed six SSRs) or, in other words, 95.0 % of all allelic variants have to be exchanged between the two species to obtain identical genetic structures.

Applying the Principle Coordinate Analysis (PCoA), *J. regia* and *J. nigra* individuals were clearly clustered in two distinct groups indicating low genetic distances between individuals within a group and large genetic distances between individuals between groups (Figure 1).

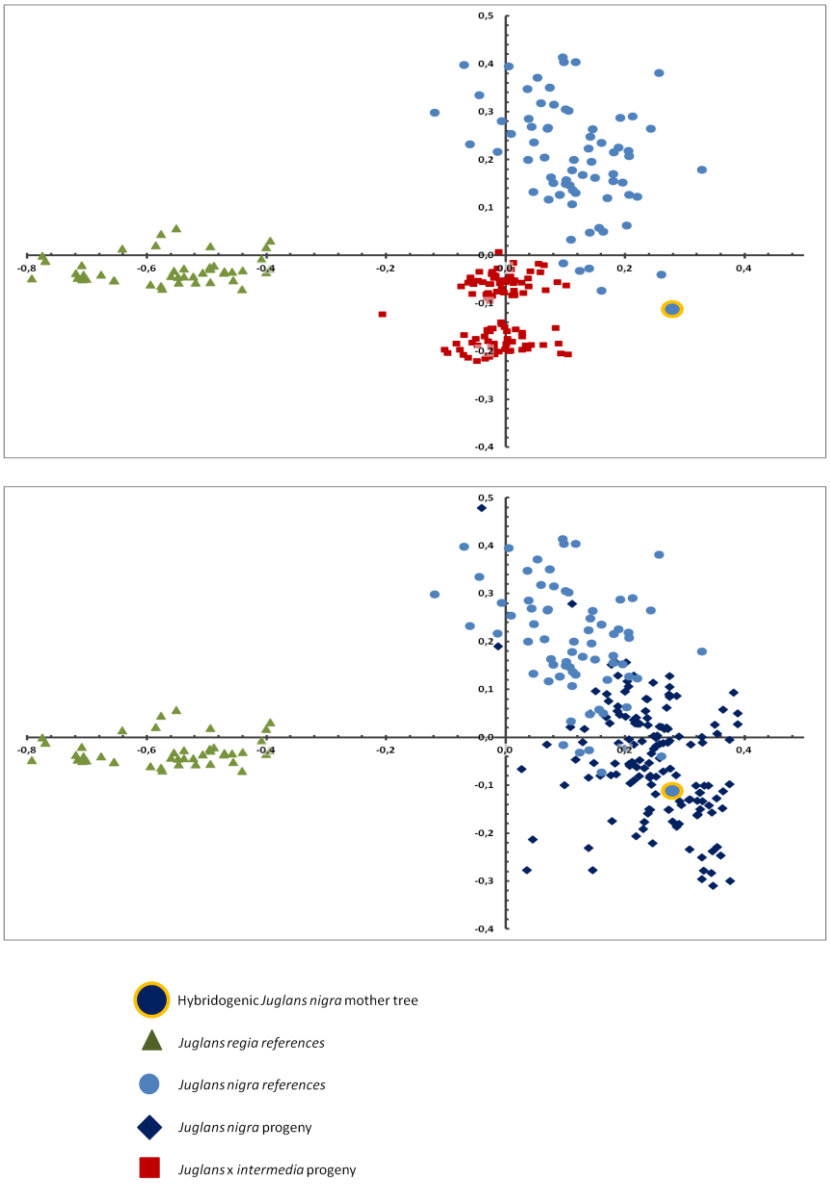

Figure 1: Individual assignment of the genotypes into groups after application of the Principle Coordinate Analysis (PCoA); both coordinate systems contain the reference collections (*J. regia* and *J. nigra*) including the hybridogenic *J. nigra* tree; the upper coordinate system contains *J.* x *intermedia* progeny; the lower coordinate system includes *J. nigra* progeny

J. regia individuals were packed more close together in the coordinate system due to lower allelic diversity and, consequently, also lower pairwise genetic distances than in *J. nigra*.

An interesting observation was the large (Eukledian) genetic distance between the hybridogenic mother tree and the analyzed reference samples of *J. nigra* as an effect of rare allele combinations. This led to an outgrouping of this individual in the PCoA-coordinate system. The *J. nigra* progeny is grouped around the hybridogenic tree, accordingly.

J. x *intermedia* hybrids are located, as expected, between the pure species *J. regia* and *J. nigra*. Interestingly, the walnut hybrids form two genetically distinct groups in close vicinity to each other. Altogether, the progeny of our hybridogenic tree can be devided in 155 *J. nigra* and 108 *J.* x *intermedia* individuals.

Genetic structures of progeny and its siring pollen

Genetic diversity: The genetic diversity of the progeny and the associated pollen contributions which successfully pollinated the hybridogenic *J. nigra* tree is shown in Table 3. One part of the progeny represents a *J. nigra* half-sib family based on intraspecific crosses and the other part a hybrid half-sib family (*J.* x *intermedia*) based on interspecific crosses with *J. regia*.

Table 3: Genetic diversity parameters (number of observed alleles N_A, effective number of alleles N_e) calculated for the *Juglans nigra* and *J.* x *intermedia* progeny of the hybridogenic mother tree and for the pollen contribution in the progeny.

	Progeny				**Pollen contribution**			
	J. nigra		***J.* x *intermedia***		**to *J. nigra***		**to *J.* x *intermedia***	
Locus	N_A	N_e	N_A	N_e	N_A	N_e	N_A	N_e
WGA027	13	3.639	5	3.986	13	6.970	3	2.008
WGA118	14	3.452	5	3.987	14	5.067	3	1.998
WGA089	18	3.950	6	4.511	18	8.658	4	2.732
WGA331	10	2.911	5	3.991	10	4.209	2	1.994
WGA069	9	1.380	6	2.722	9	1.926	6	3.115
WGA276	17	3.364	11	5.109	17	5.785	9	4.776
Mean	**13.50**	**2.749**	**6.33**	**3.904**	**13.50**	**3.821**	**4.50**	**2.504**

In the *J. nigra* progeny the mean number of alleles was only slightly lower (N_A = 13.50) than in the reference collection (14.33, see Table 1). However, looking at the single loci, there was not only a decline of alleles (decrease of 2 to 5 alleles at the loci WGA027, WGA118, WGA331) but also an increase of genetic variants at other loci (1 to 2 alleles at the loci WGA089, WGA069, WGA276) because of previously not detected variation in pollen donors that had not been selected for the reference data collection. The harmonic

mean of the effective number of alleles (N_e) was much lower in the *J. nigra* progeny compared to the reference collection (2.75 and 4.42, respectively), because maternal alleles in ovules contribute 50% of the allelic composition to the half-sib progeny (50% of one specific allele in the case of homozygous gene loci, 0 to 50% of each of the two alleles at heterozygous gene loci depending on segregation rates).

The *J.* x *intermedia* progeny consisted of maternal alleles (see above) and the alleles inherited by *J. regia* as pollen donors. Because of the generally lower genetic diversity of *J. regia*, the total number of alleles was lower than in the *J. nigra* progeny. The effective number of alleles (N_e = genetic diversity) on the other hand was higher compared to the *J. regia* reference collection as well as the *J. nigra* progeny because of the genotypic combination of mostly private (=species specific) alleles. This led to a high individual heterozygosity of almost 100% (as is typical for interspecific hybrids with different evolutionary histories) effecting N_e due to the relationship $N_e = 1/(1 - H_e)$.

Differences were also observed between the genetic variability of the pollen contributions compared to the reference collections. Although there was an only slight decrease in the total number of alleles in the male gametes contributing to the *J. nigra* and *J.* x *intermedia* progenies, the effective number of alleles was lower in successful *J. nigra* pollen (3.82 in the pollen and 4.42 in the reference material) but, otherwise, higher in *J. regia* pollen (2.50 in the pollen and 2.16 in the reference collection).

The frequency distributions of individual heterozygosities are shown in Figure 2.

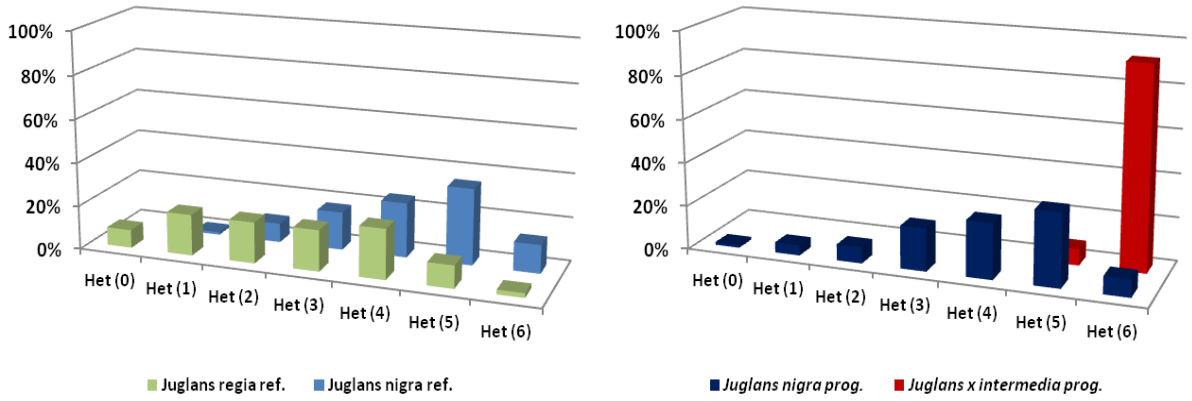

Figure 2: Frequency distribution of individual heterozygosities at six SSR marker loci (1-6) for the reference collections of the pure species *Juglans regia* and *J. nigra* (diagram on the left) and for the progeny of the hybridogenic *J. nigra* mother tree divided in *J. nigra* and *J.* x *intermedia* progeny (diagram on the right)

The *J. nigra* progeny of the hybridogenic tree represented almost the same profile than the Black walnut reference collection with a slight tendency to lower individual heterozygosities (average values of 3.97 and 4.23, respectively). The *J.* x *intermedia* progeny, however, was heterozygous for 5.93 of the six analyzed loci, on average.

Allelic profiles: Figure 3 illustrates allelic frequencies of the contribution of successful *J. nigra* pollen to the Black walnut progeny. Most of the frequent alleles found in the reference data set can also be found in the pollen cloud. Two aspects are worth mentioning:

1. At the SSR-locus WGA027 two alleles (215 and 237) were the most frequent ones in the contributing male gametes but occurred with only very low frequencies in the reference collection. This is a clear indication of a considerable proportion of self-pollination because the hybridogenic mother tree shared exactly this combination of alleles. The same situation holds true for locus WGA089. In contrast to the *J. nigra* reference material, allele 220 displayed the second most frequent allele in the pollen contribution and appeared in the heterozygote combination 212-220 bp also in the maternal tree (marker WGA089, Figure 3).

2. *J. regia* specific private alleles occurred at very low frequencies also in the *J. nigra* progeny (see alleles 197 bp at locus WGA118; 218 and 222 bp at locus WGA089; 160, 180 and 182 bp at locus WGA069; 178, 190, 192, 194 bp at locus WGA276; Fig. 3). Thus, backcrosses from existing *J.* x *intermedia* trees in the surrounding of the hybridogenic mother tree cannot be excluded.

Figure 4 shows allelic frequencies of the contribution of successful *J. regia* pollen to the *J.* x *intermedia* hybrid progeny. In accordance to intraspecific matings, also in these interspecific crosses the most frequent alleles could be found in both the pollen contribution as well as the reference collection of *J. regia*. Rare additional alleles in the pollen contributions could be observed at four loci (WGA027, WGA089, WGA069, and WGA276), whereas several alleles are also missing in the pollen (also four loci: WGA118, WGA331, WGA069, and WGA276).

At least five pollen donors (but probably more) must have contributed to the *J.* x *intermedia* progeny assuming heterozygote pollen donors for locus WGA276 (N_A = 9).

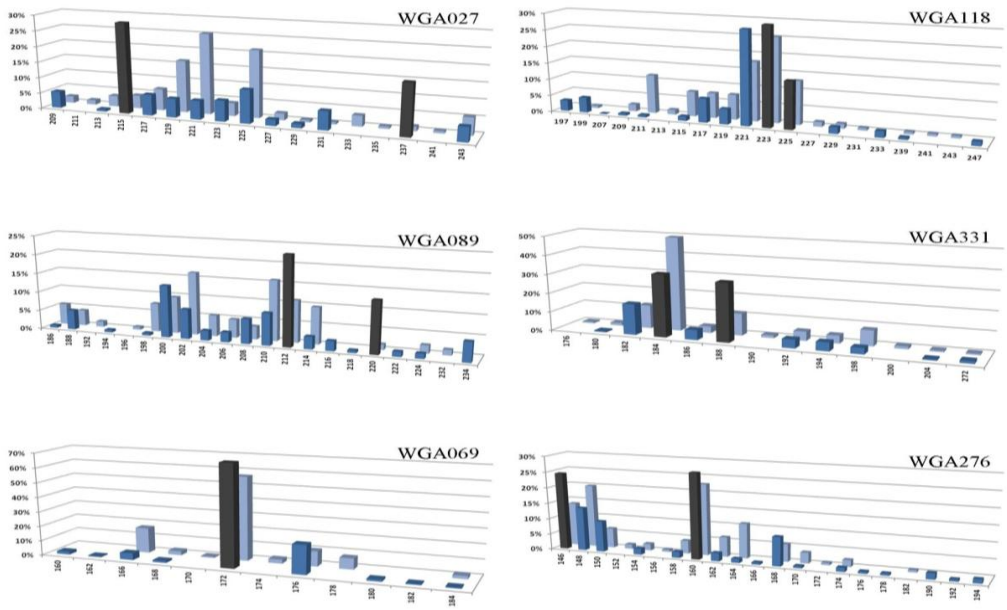

Figure 3: Allelic profiles of the *J. nigra* pollen contribution (intraspecific mating) to the progeny of the hybridogenic mother tree (dark blue bars in the foreground) compared to the allelic profiles of the *J. nigra* reference collection (light blue bars in the background); dark grey bars represent the allelic combination of the hybridogenic mother tree.

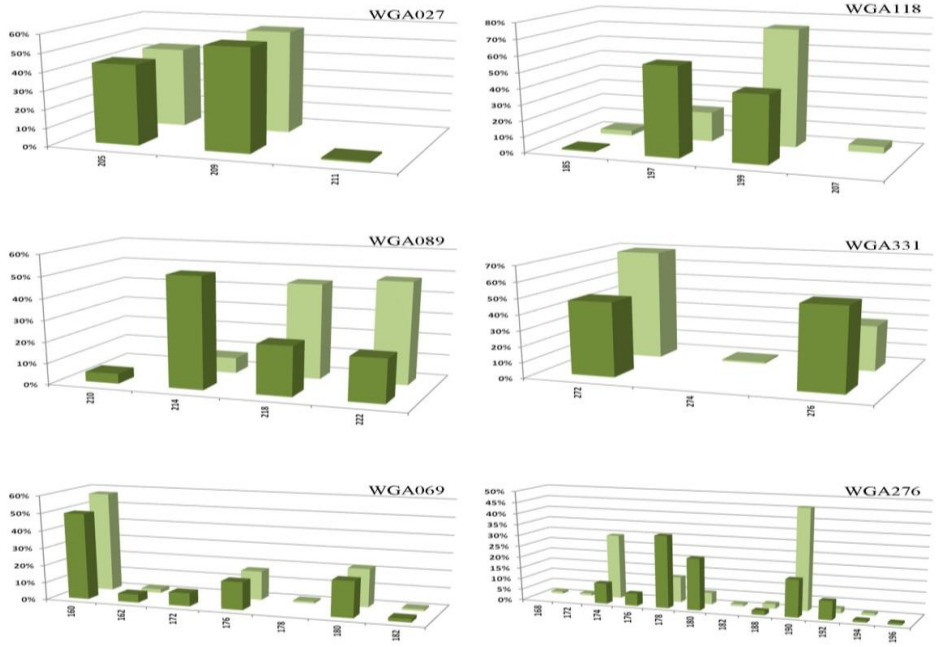

Figure 4: Allelic profiles of the successful *J. regia* pollen contribution (interspecific mating) to the progeny of the hybridogenic mother tree (dark green bars in the foreground) compared to the allelic profiles of the *J. regia* reference collection (light green bars in the background)

Self-fertilization vs. outcrossing

For our analysis we used LOD scores (log-odds ratio), which represent the ratio of the likelihood of a *J. nigra* individual being the father (pollen donor) of a given offspring and the likelihood that the potential pollen donor and the offspring are unrelated. After evaluation of all genetically possible father trees, offspring were assigned to candidate fathers with the highest, positive LOD score. In our study, mating partners could be assigned for 66 and 71 of the 155 *J. nigra* offspring individuals, depending on the estimated number of totally recorded potential pollen donors of 70 or 90%, respectively (Figure 5).

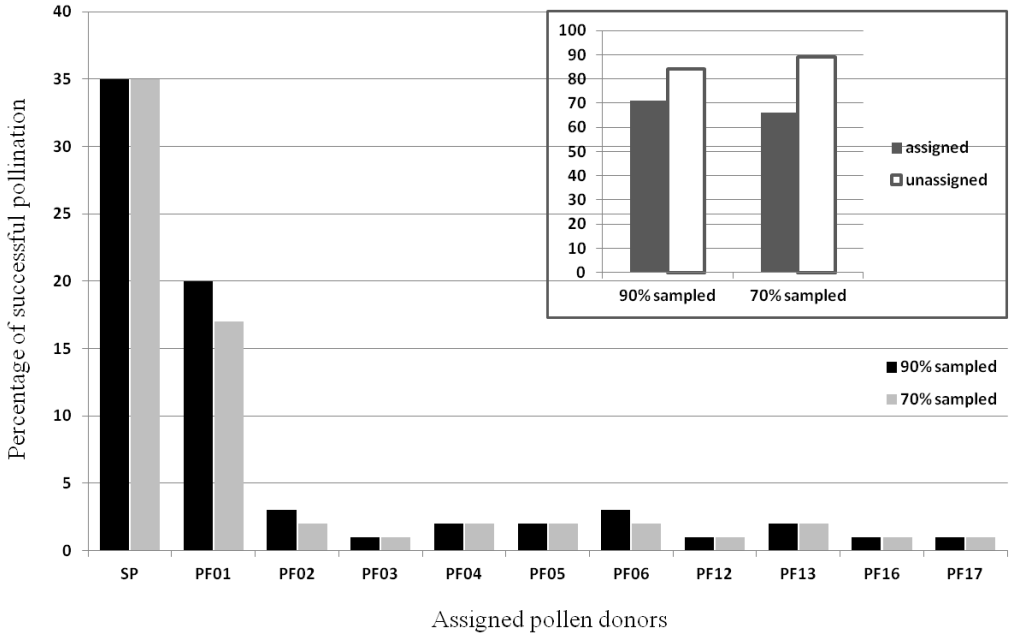

Figure 5: Most likely assigned candidate fathers of intraspecific matings of the hybridogenic mother tree (*J.nigra*); SP = self-pollination, PF01 to PF17 = most likely potential fathers (= pollen donors) in the surrounding of the hybridogenic tree; box top right: number of assigned (highest, positive trio LOD scores between offspring and candidate parent and a confidence ≥ 0.8) and unassigned progeny (negative trio LOD and a confidence < 0.8); calculations assume that 70% or 90% of all existing potential pollen donors have been sampled

On the basis of this estimation procedure, self-pollination played an important role in the in intraspecific matings of this single hybridogenic tree. More than 20 % of the progeny is the result of self-fertilization (35 out of 155 individuals of the *J. nigra* progeny). Outcrossing was estimated on less than 80%. Only about 35 to 43% of the pollen contributions could be

assigned to ten of the recorded potential father trees, the rest of potential pollen donors remained unassigned. The *J. nigra* tree PF01, only about 100 m distant from the hybridogenic tree, displayed to be the most important pollinator for intraspecific outcrossings.

Considering interspecific matings, the two potential *J. regia* fathers (distance about 0.1 km), flowering simultaneously with the hybridogenic mother tree (observations according to Anton Schott), turned out to be pollen donors for only 15% of the *J.* x *intermedia* progeny (with a strict confidence threshold of 0.95). For this first estimate we used allele frequencies of all reference data for simulation analyses to establish a confidence threshold for assignment.

Discussion

Activities in hybrid tree breeding and clonal forestry have strongly increased, particularly to meet the expanding demands of the bioenergy and paper pulp industry. In temperate and boreal zones controlled interspecific crossings are mostly applied to species of the *Populus* and *Salix* genera (e.g. DICKMAN & STUART 1983, GEBHARDT et al. 2012, JANßEN et al. 2008, JOACHIM 1991, LIESEBACH et al. 1999, PEI et al. 2008, YU et al. 2001), but also tropical countries are adopting hybrid breeding for industrial plantations (e.g. *Eucalyptus*, SUBASHINI et al. 2014).

In the last decades, hybrid walnuts (*Juglans* x *intermedia*), achieved by crossings between American black walnut (*J. nigra*) and Persian walnut (*J. regia*), have raised interest. These hybrids are of particular interest for valuable timber production being an excellent compromise between growth rate, resistance to damaging agents and climatic uncertainties. Heterosis allows harvesting timber of high quality already after 50 to 70 years according to site conditions. Due to this rapid development and vigorous competition *J.* x *intermedia* is suitable not only for plantation forestry but also for cultivation as single trees or groups in mixed deciduous forests (EHRING & KELLER 2006). In the latter case hybrid walnut probably represents an interesting alternative to *Fraxinus excelsior* (Common ash) suffering from ash dieback having devastating consequences for the species survival on large European scales (THOMSEN & SKOVSGAARD 2012).

The increasing demand for *J.* x *intermedia* is accompanied by a low availability of reproductive material. Only a very few open pollinated "hybridogenic" Black walnut mother trees are known producing hybrid walnut progeny and controlled pollination is still

difficult due to embryo abortion, pistillate flower abscission caused by excess of pollen load and low viability of stored walnut pollen, so that the production of hybrid plants depends mostly on successful natural hybridization (MCGRANAHAN et al. 1994, KRÜGER 2000, POLLEGIONI et al. 2010, JAFARI-MOFIDABADI 2014).

However, in the few existing field trials survival between different hybrid genotypes proved to be highly variable (CLARK & HEMERY 2010). Thus, it would be reasonable to produce multiple *J.* x *intermedia* clones with a sufficient broad genetic base by vegetative propagation to start improvement programs on differing ecological sites in order to select elite genotypes for further use in forestry. But, embryo axes from mature seeds turned out to be the best appropriate tissue to be used for an efficient large-scale micropropagation system. Micropropagation of existing mature hybrid trees is also possible, but much more difficult and less efficient (MEIER-DINKEL, in press). In several other studies *in vitro* `embryo rescue techniques´ have been efficiently used to overcome incompatibility barriers during the development of interspecific hybrids, e.g. in tree and fruit breeding programs (RAMMING 1990, JAFARI-MOFIDABADI et al. 1998, JAFARI-MOFIDABADI 2014). Thus, the combination of DNA based tools for the early identification of *Juglans* x *intermedia* hybrids in seed material and optimized *in vitro* propagation methods using embryo material has proved to be an essential basis for the production of a multiclonal variety of plants to be tested in field trials before application in forestry (TUAN et al. 2016). Our results show that a precise genetic differentiation between the two species and their hybrids is possible with an already limited number of markers. In this study, the genetic distance parameter d_0 (GREGORIUS 1978) represents very high values between the reference sets for *J. regia* and *J. nigra*, indicating that only 0.5 to 10.3% of the allelic composition is shared by both species (Table 3). On this basis, several population genetic standard programmes (STRUCTURE, Principle Coordinate Analysis [PCoA] etc.) can be applied for species discrimination (see also POLLEGIONI et al. 2009a, 2009b).

The genetic structure of the progeny of single hybridogenic trees is basically dependent on the genetic variability that is available in potential mating partners. Progeny from open pollination can be the result of A) intraspecific crosses with surrounding *J. nigra* trees (half-sib or ful-sib-families as a result of outcrossing) or with self-pollen (self-fertilization) as well as B) interspecific crosses with surrounding *J. regia* trees.

According to POLLEGIONI et al. (2017) a clear longitudinal trend of walnut genetic diversity in Eurasia was found with a marked loss of allelic diversity running from eastern

to western Europe as a result of human dispersal and exploitation activities over the last 5,000 years and population expansion / contraction from multiple refugia after the last glacial maximum. The genetic diversity of the German *J. regia* reference set in this study with a mean total and effective number of alleles of N_a = 4.67 and N_e = 2.16 corresponds to the values found in other populations of Southern and Western Europe. In populations of France, Italy, Spain and Hungary these values range from 3.33 to 4.50 and 2.13 to 3.09, respectively. In the Balkan and western Asia the above mentioned diversity parameters reached distinctly higher values from N_a = 4.50 to 6.00 and N_e = 2.52 to 4.16. The latter data are based on recalculations using the same set of microsatellite markers from supplementary raw data according to POLLEGIONI et al. (2017), including populations with more than 20 individuals only.

According to BARTSCH (1989), *J. nigra* was introduced to England in the 17th century as single trees and planted in botanical gardens and parks. From England this tree species was further distributed to other European countries (France, The Netherlands, Germany). In the second half of the 19th century first silvicultural field experiments were started. Thus, the German *J. nigra* reference set is a collection of individuals that presumably descend from a smaller number of seed trees. This assumption is supported by higher genetic variability found in its natural range of distribution of North America. At the commonly studied SSR-loci WGA027, WGA069 and WGA089 VICTORY et al. (2006) observed a total of 68 alleles (25, 14 and 29 alleles, respectively), whereas only 42 alleles (each locus with 18, 8 16 alleles) can be observed in the German reference set (Table 2).

Overall, the genetic diversity was, in accordance with former studies (POLLEGIONI et al. 2009a, 2009b), markedly higher in the *J. nigra* than in the *J. regia* reference set.

The genetic composition of the progenies of the hybridogenic mother tree (a. *J. nigra* and b. *J.* x *intermedia* offspring) reveals differentiating results:

a) The analyzed *J. nigra* offspring shows a distinctly lower overall genetic diversity than the reference material (Table 3). This is not only based on the restricted number of alleles contributed by the mother tree as is typical for half-sib families (one allele at the homozygous locus WGA069 and two alleles at the remaining heterozygous loci) but also due to the lower allelic variation in the pollinating pollen cloud regarding the total and effective number of alleles (N_a = 13.5 and N_e = 3.82, respectively, Table 3). Further, the allelic profiles of the pollen contribution and the conducted paternity analysis provide clear

indications on self-fertilization rates of more than 20 %. This is exceptionally high compared to observations in populations of its native range (outcrossing rates ranging from 90 to 100%, see BUSOV et al. 2002). Based on these interesting findings, continuing investigations are necessary considering a combination of a DNA based analysis of mating relations and observations on the flowering phenology of this hybridogenic mother tree and its surrounding *J. nigra* and *J. regia* trees (see POLLEGIONI et al. 2012). Although *J. nigra* as well as *J. regia* are characterized by dichogamous flowering, the period of female flower receptivity of the hybridogenic *J. nigra* tree seems to overlap with the period of mature pollen release of both, its own male flowers as well as male inflorescences of surrounding *J. regia* and *J. nigra* trees. Our study confirms former investigations that the temporal separation of male and female flower bloom of walnuts walnuts is sometimes incomplete (see FORDE & GRIGGS 1975, POLLEGIONI et al. 2010)

b) *J.* x *intermedia* offspring mostly combines species specific (private) alleles leading to high degrees of individual heterozygosities. More than 90% of the hybrid individuals are found to be heterozygous for all six analyzed gene loci compared to 8.1% in the *J. nigra* half-sib progeny and 2.1% and 13.0% in *J. regia* and *J. nigra* reference material, respectively (Fig. 2). This finding is most likely the result of different evolutionary histories of the two parents leading to the observed heterotic effects (KAIN 2003).

The *J. regia* pollen contribution shows a slightly higher genetic diversity than the reference set as well as the studied populations of Southern and Western Europe (POLLEGIONI et al. 2017). At two loci WGA069 and WGA276 we observed much higher diversity values (3.11 and 4.78 effective alleles, Table 3), assuming that diverse pollen donors have contributed to the genetic composition of the *J.* x *intermedia* progeny resulting in a rather broad genetic base for further use of the progeny in field trials. Additional paternity tests are aspired to find out the almost complete set of *J. regia* fathers of this hybridogenic tree in order to assemble an effective *J.* x *intermedia* breeding population (seed orchard based on grafted plants).

Further, more hybridogenic trees should be detected in order to enlarge the genetic base and adaptability of walnut hybrids to changing environmental conditions. Until now, about 55 *J.* x *intermedia* hybrids from this one hybridogenic tree (RENI) are micropropagated for further testing on different planting sites.

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3. Discussion

Due to the highly valuable timber for veneer and solid wood, the price of the *Juglans* species, particularly *Juglans* hybrids, has been increasing not only on the European market but also on the global market. However, the production of the *J.* hybrid plants, which are expensive and rare, up to now, depends mostly on successful natural hybridization (Pollegioni et al. 2010), and the amount produced is not sufficient for afforestation and plantations. These reasons have led to many studies dealing with ways to alternative propagation during the last three decades.

Two major aspects were studied in this thesis. One focused on the potential composition of a multiclonal variety of *J.* x *intermedia* that is suitable for high-value timber production in forest plantations and concentrated on the following two aspects: (1) the identification and characterization of interspecific *J.* x *intermedia* embryos from an open pollinated tree and (2) the optimization of a tissue culture protocol for *Juglans* genotypes for potential application in commercial large-scale propagation.

The other part focused on the endophytic bacteria and their role in improving the micropropagation of *Juglans*.

3.1 Production of a multiclonal variety of *J.* x *intermedia* for silviculture and plantation forestry.

Identification and characterization of interspecific J. x intermedia embryos from an open pollinated tree by microsatellites.

To our knowledge, both the identification and characterization of the German interspecific hybrid (commercialized as RENI) by molecular techniques have not been studied or mentioned in the literature. There are only a few studies by the Forestry Research Institute in Freiburg, Germany with promising results (Ehring et al. 2009). However, most studies have focused only on the limiting factors, e.g., site condition, the limited quantity of plants, and seeds from appropriate provenances for valuable wood production.

Recently, more attention has been paid to the identification and selection of offspring for *Juglans* hybrids from hybridogenic parent trees with encouraging results. The genetic structure of the progeny of single hybridogenic trees is dependent on the genetic variability that is available in potential mating partners. Microsatellites [= simple sequence repeats methods (SSRs)], were considered suitable tools for both parentage analysis due to high

allelic variability (Gillet 1999, Streiff et al. 1999) and species and hybrid identification due to high genetic distance between species. Considering the results of the PCoA (=Principle Coordinate Analysis) based on genetic distance parameters, the *Juglans* hybrid genotypes are clustered between the *J. nigra* and *J. regia* genotypes, which share the allelic composition of both of them, permitting the identification of new interspecific hybrids and establishing new seed orchards for hybrid production (Pollegioni et al. 2010). The results of this study indicate that *J.* x *intermedia* offspring mostly combines species specific (private) alleles leading to high degrees of individual heterozygosities. More than 90% of the hybrid individuals from an open pollinated tree are found to be heterozygous for all six analyzed gene loci (WGA027, WGA118, WGA089, WGA331, WGA069, WGA276). Thus, application of microsatellites in combination with micropropagation techniques can result in a rapid and economic identification of new hybrid clones for forest plantations (see 2.2 and manuscript 2.4).

Another possibility for identifying interspecific walnut hybrids was the choice of the establishing medium. In the presented research, the best combination for shoot elongation and establishment of shoot cultures in vitro was 4.4 µM BA and 0.005 µM IBA in modified Rugini (Rugini 1984) or DKW (Driver and Kuniyuki 1984) media. Under the same conditions, *J. nigra* genotypes elongated less (Fig.7), expressed more chlorosis and died after several weeks. The hybrids selected during the establishment phase were later verified by microsatellites. Thus, these results provided a useful identification of *Juglans* hybrids and *Juglans nigra* in micropropagation. Furthermore, in our study at the NW-FVA (data not shown) the best combination plant growth regulator for *J. nigra* shoot elongation and in vitro establishment was 17.5 µM ZEA and 0.005 mg/l IBA in Rugini (Rugini 1984) media. These results are in agreement with a report by Bosela and Michler (2008) that ZEA was superior for *J. nigra* shoot elongation than BA.

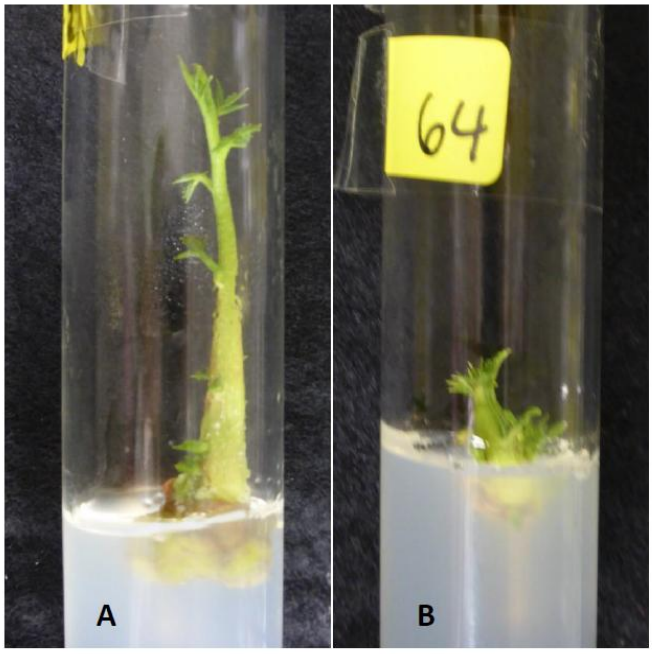

Fig.7: Shoot development from embryo axes of *J.* x *intermedia* (A) and *J. nigra* (B) after 20 days of culture with 4.4 µM BA and 0.005 µM IBA in modified Rugini medium in test tube 16 x150 mm.

According to Pollegioni et al. (2010) the difficulty in obtaining hybrids between *J. nigra* and *J. regia* could be the temporal separation of male and female flower bloom. In our findings, the period of female flower receptivity of the hybridogenic *J. nigra* tree seems to overlap with the period of mature pollen release of both species (see manuscript 2.4). However, this result also requires further analysis by observations on the flowering phenology of this hybridogenic mother tree and its surrounding *J. nigra* and *J. regia* trees.

Multiclonal hybrid variety and the selection of candidates for genotype varieties for forest plantations

Multiclonal varieties are a mixture of many genotypes as a result of outbreeding and successful sexual reproduction could be more ideal than monoclonal varieties for forest tree improvement (Zsuffa et al. 1993). Due to rapid development and vigorous competition, *J.* x *intermedia* is not only suitable for plantation forestry but also for cultivation as single or groups of trees in mixed deciduous forests (Ehring & Keller 2006). The research presented here will contribute to the establishment of a multiclonal hybrid variety through determination of genetically superior genotypes in clonal field trials for quality and growth performance.

According to Clark & Hemery (2010), only a few of highly variable hybrid genotypes proved to survive in field trials. However, 55 *Juglans* hybrid genotypes, which originated

from an open pollinated tree, would be reasonable to produce multiple *J.* x *intermedia* clones with a sufficient broad genetic base by vegetative propagation to start improvement programs on different ecological sites to select elite genotypes for further use in forestry. Genetic variation and allelic profiles of the successful *J. regia* pollen show that diverse pollen donors have contributed to the genetic composition of hybrid progeny resulting in a broad genetic base for further use in field trials. Furthermore, a large number of these *Juglans* hybrids and *J. regia* genotypes also have been tested regarding their growth behaviour in our greenhouse, and the results could be useful for large-scale production of *Juglans* hybrids for practical forestry.

Optimization of the in vitro propagation of the Juglans *species and potential applications for commercialization*

The increasing demand for *J.* × *intermedia* wood from industries on the world market has led to the expansion of planting areas and the establishment of new genotypes for afforestation. However, some of the offspring from hybridogenic parent trees are not really interspecific hybrid genotypes (McKay 1965; Scheeder 1990). The proportion of hybrids in progenies from natural sources is usually less than 10%, and their germination rate is relatively low (may be as low as 27 percent) (Funk, 1970). Thus, to produce a multiclonal *Juglans* hybrid variety, using tissue culture appears to be the most effective method for large-scale propagation.

In this study (see 2.2), we optimized the procedures for the micropropagation of the *Juglans* hybrids. Using embryo axes cultures, our results indicated that the obvious growth of the *Juglans* hybrids was faster and stronger than that of the mature explants which were much more difficult and less efficient (Meier-Dinkel et al. 2017). Both the Rugini (Rugini 1984) and DKW (Driver and Kuniyuki 1984) media appeared to be suitable for shoot multiplication of *J.* x *intermedia.* Furthermore, comparing the three gelling agents (Oxoid agar, Kobe agar, and Gelrite), Kobe agar has been proven to be better than the other solidifying compounds in the *J. x intermedia* shoot cultures. These results are important for both future studies and commercial micropropagation because the price of Kobe agar is three times cheaper than that of Gelrite.

Regarding the propagation rate within the scope of this study, we observed that there were more than 4.0 new sub-culturable explants per explant after the 21-day sub-cultures. This is, indeed, a high rate of multiplication. It is, therefore, very likely that this is one of the most effective and easiest approaches to reduce the length of the breeding cycle through

the use of juvenile plant material with characteristics like rootability and fast orthotropic growth when establishing new *Juglans* hybrid genotypes based on a limited supply of nuts from the hybrid parents (see 2. 3).

The problem of decreasing rooting success not only in adult woody plants but also in microcuttings is still unsolved (Winkelmann 2012), particularly for *Juglans* species. A recently published article (Pollegioni et al. 2010) noted that walnut hybrids are difficult to propagate with a limited ability to form adventitious roots. Furthermore, according to Jay-Allemand et al. (1992), the factors affecting rooting in the micropropagation of the *Juglans* species depend not only on internal conditions, e.g., genetic, morphological or physiological but also on environmental conditions, e.g., light, temperature, and moisture. The results of this study indicated that by modifying the technique proposed by Jay-Allemand et al. (1992), the rooting ability of the *Juglans* species was improved by two-phase rooting, including an induction and an expression phase.

The rooting ability of the *Juglans* hybrids can also be improved by using darkness as an essential factor for the root induction. Also, the combination of Vermiculite and Gelrite appeared to provide a good balance among aeration, the availability of water (humidity) and nutrients that are reliable substrates for the outgrowth of roots of the *Juglans* hybrids. Thus, the interactions between the genotype and the environment caused the effect of the *Juglans* hybrids rooting. Furthermore, the rooting capacity reduces as the age of the source of explants increases (Rodriguez et al. 1989). However, with the experience gained nearly four years indicates that rooting in *Juglans* microcuttings was not a great problem when healthy tissues are used (the length of *Juglans* microcutting not less than 2 cm) and improved air/ water ratio which allowed for superior rooting success. The present study aimed to identify the optimal technique for promoting root development and enhanced the rooting rates (from 50 to 73%), and the survival rates after the acclimatization were 75 to 93% (see 2.2 section, Fig 7).

Fig. 8: Key stages in micropropagation procedure for the *Juglans* hybrids. *A: Juglans* hybrid shoot culture; B: Rooted plantlets transplanted; C: Tissue-cultured plants after one year in the nursery.

3.2 Endophytic bacteria and possible impact on micropropagation of *Juglans*

In our study, the endophytic bacterial community structure in the *in vitro* shoots was assessed using culture-dependent and culture-independent methods. Regarding the culture-dependent method, a total of eight endophytic bacterial strains with a high identity score (97-100%) was isolated from the shoot cultures of the *Juglans* hybrid material. However, no bacteria were isolated from three *J. nigra* and two *J. regia* genotypes during the five weeks of observation. Furthermore, using the culture-independent approach, Illumina MiSeq 16S rRNA amplicon sequencing, which was considered to be not only used for environments with high diversity but the most suitable method for the low diversity of the bacteria in the micropropagation, more than 85% of the total population of the bacteria were detected in all analysed samples. Meanwhile, the genera of bacteria isolated in the bacterial culture of the 16 *Juglans* genotypes, including *Acinetobacter*, *Moraxella*, *Brevundimonas*, *Pseudomonas* and *Roseomonas*, were also detected by Illumina MiSeq in the same genotypes (see Manuscript 2.3).

The origin of the detected bacteria is not known, they have been introduced by the source material or by human handing. However, it seems that the bacteria derived from the original plant tissue, because not only the embryonic axes from *Juglans* nuts were always carefully sterilized, but the explants were also prepared aseptically in all micropropagation procedures. Furthermore, some endophytic bacteria persist in the nuts and are inherited by vertical transmission over several host plant generations (Laukkanen et al. 2000, Koskimäki et al. 2015). The presence of endophytic bacteria in meristems, flowers and seeds has been shown in other studies, e. g. for *Methylobacterium extorquens* in Scots pine (Koskimäki et al. 2015) and for *Methylobacterium* and *Pantoea* species frequently been detected in seeds of diverse plant species (Mano and Morisaki 2008; Johnston-Monje and

Raizada 2011). However, the *Juglans* microshoots from embryonic axes used in this thesis had been subcultured for 2 to 4 years at the time of bacterial analysis. Therefore, in future, embryonic axes should be used to identify populations of bacterial species, and the culture independent approach (Illumina MiSeq sequencing) would be the suggested method. Contamination by human handling which can be spear from one plant to the next, on the other hand, would most likely introduce one strain to many genotypes during the transfer of plantlets. However, the composition of bacteria detected in different *Juglans* genotypes was differing between genotypes.

Detection of bacterial communities by NGS

There are several molecular fingerprinting techniques that can be used to analyse endophytic bacterial communities, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (TRFLP), temperature gradient gel electrophoresis (TGGE) and amplified ribosomal DNA restriction analysis (ARDRA), (Garbeva et al. 2001; Abreu-Tarazi et al. 2010; Videira et al. 2013; Quambusch et al. 2014). However, next-generation sequencing (NGS), which is known as high-throughput sequencing and includes 454 Pyrosequencing or Illumina MiSeq, has revolutionized the study of molecular biology and is commonly used to detect microbial communities (Oberauner et al. 2013; Uroz et al. 2013; Hong et al. 2015).

According to Knief (2014), Illumina MiSeq 16S rRNA amplicon sequencing can detect much lower abundant taxa due to the greater depth of the analysis compared with denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), which were widely used in analyses of endophyte communities. Furthermore, based on the development of nucleic technology, nanotechnology, and microscopy, Illumina MiSeq can detect millions of small sequence reads simultaneously. Thus, DNA sequencing becomes rapid and cheap (Hudson 2008). However, the NGS reads only provide resolution at the genus level (Knief 2014), while Sanger sequencing, in most cases, could identify bacteria at the species level.

Hudson's study in 2008 described NGS with three technologies. First, the pyrosequencing 454 method was developed by 454 Life Sciences, Roche (Branford, CT, USA) using pyrophosphate release (known as the pyrosequencing method) for the detection of base incorporation (Ronaghi et al. 1996; Rongahi 2001) which is capable of generating 80-120 Mb of sequencing in 200-300 bp reads (Morozova et al. 2008). Second, Illumina technology, known as ‘1G Genome Analysis System’, was developed by Solexa (Hayward,

CA, USA) by applying 'sequencing via synthesis' (SBS) technology, which could be used to detect microbial taxa with low abundant species changes (Oberauner et al. 2013; Uroz et al. 2013). Illumina technology is capable of producing at least 1Gb of sequencing and generating 35 bp reads (Morozova et al. 2008). Finally, the ABI SOLiD (Sequencing by Oligonucleotide Ligation and Detection) method from Applied Biosystems has recently become available, based on 'polony sequencing' technology and the sequencing chemistry developed by Shendure et al. (2005), this instrument is capable of producing 1-3 Gb of sequencing data and generating 35 bp reads (Morozova et al. 2008).

Compared with Pyrosequencing 454, the Illumina sequencing method is more cost effective (approximately one-twentieth to one-thirtieth) and can obtain more sequences per sample (at least 1G (one gigabase) bases per run compared to 80-120Mb bases per run) (Hudson 2008; Kozich et al. 2013), and the base-call error, contig length and frame shift were also evaluated as better than those in 454 Pyrosequencing (Luo et al. 2012).

In the presented research, although primer 799f was selected, which avoided the amplification of chloroplast DNA sequencing by a specific mismatch primer instead of the universal bacterial primers during the PCR amplification (Chelius and Triplett 2001), the results of the NGS (Illumina sequencing) showed a high percentage of chloroplast (54.8 - 88.4%) and mitochondrial (7.0 - 30.5%) sequences in our plant samples. The results were also confirmed by Bodenhausen et al. (2013), who showed that the primer 799f amplified bacterial and plant chloroplast DNA (approximately 62% of the sequences). However, the primer 799f has been used successfully in many studies. For example, Quambusch et al. (2014) have been able to successfully reduce the impact of contaminating sequences on *Prunus avium* tissue cultures and found only 1 of the 380 sequences assigned to chloroplast DNA, while no mitochondrial fragments were identified.

Future studies should employ quantitative PCR analyses to investigate bacterial concentrations in the plant tissues. In samples with very low bacterial DNA content, the development of blocking primer, which could further reduce the chloroplastic and mitochondrial sequences to reach higher coverage than bacterial sequences. Arenz et al. (2015) reached a 300-fold increase of bacterial reads with blocking primers compared to standard PCR in an Illumina-based study of *Sorghastrum nutans* leaf endophytes or by reducing the amplification of sequences using suicide polymerase endonuclease restriction (SuPER) (Green and Minz 2005), peptide nucleic acid (PNA)-mediated PCR clamping (Lundberg et al. 2012), which could be suitable for samples with very low bacterial

concentration as embryo axes materials, and another method to reduce the co-extraction of organelle DNA by modifying the DNA extraction protocols (Lutz et al. 2011).

3.3 Outcome of the study

1. Identification and characterization of the interspecific J. x intermedia *embryos from an open pollinated tree.*

J. x *intermedia* has already been widely planted in forest plantations in Europe as timber tree (Woeste and Charles 2011). However, walnut hybridization is rare under natural conditions; hence, the development of a protocol for the micropropagation of the *Juglans* hybrids has important implications. Microsatellite (SSR-) markers are analyzed to provide information about the genetic diversity of the progeny as a basis for improvement and field testing programs as well as for early selection and use in propagation of superior genotypes in production (plantation) forestry. The potential applications of this thesis could provide a basic tool for the production of a multiclonal variety of *Juglans nigra* x *J. regia* hybrids (*J.* x *intermedia*) by employing DNA-based techniques for the early identification of hybrid genotypes. The first beneficial outcome of our research is the establishment of a set of 55 new verified genotypes of *J.* x *intermedia* (furthermore eight *J. regia* and six *J. nigra* genotypes) from six microsatellites WGA027, WGA118, WGA089, WGA331, WGA069, WGA276 (see manuscript, section 2.2 and 2.4)

2. Optimization of the in vitro *propagation of the* Juglans *species*

Although the *Juglans* shoots growth and rooting rate appear to be very dependent on the varieties, the protocol for *Juglans* axillary shoot multiplication was improved not only for *J. regia* but also for *Juglans* hybrids by evaluating different culture media and varying the nutrient composition, cytokinin concentration, and gelling agents. Moreover, the low rooting rates remain the limiting factor in the micropropagation of the *Juglans* species. In this thesis, the highest rooting percentages of microcuttings ranged from 50 to 73%. Furthermore, more than 75% of the plantlets continued to grow vigorously during the acclimatization in the greenhouse by the proposed method (see manuscript, sections 2.1 and 2.2).

3. Analyzing the bacterial community structure in tissue cultures of three main Juglans *genotypes and the possible application of the endophytes in the micropropagation of the* Juglans *species.*

Another beneficial outcome of this study is the gained knowledge about the diversity and first insights into the mode of action of endophytes in tissue cultures of the *Juglans* species. While a diversity of endophytic bacteria from tissue culture shoot materials derived from embryo axes of the *Juglans* species was recorded using culture-dependent and culture-independent methods. In addition, the abundance of the bacterial community structure was also detected by culture-independent methods, and some of these bacteria are known to be plant growth promoting bacteria, such as *Pseudomonas* sp., *Erwinia* sp., *Burkholderia* sp., *Pelomonas* sp., and *Sphingomonas* sp.. Furthermore, *Moraxella* sp., a possibility would be the introduction of the beneficial strain, accounted for genotypes with optimal propagation rates, and it could be inoculated as an endophytic bacterium to shoot cultures to test its potential beneficial effects on micropropagation (see 2.3)

3.4 Outlook

3.4.1 Future prospects for rooting rate improvement and potential application of endophytes in micropropagation

Propagation success is strongly dependent on the genotype and shows a high fluctuation over time (C. Schneider, personal communication). In this thesis, the *J.* hybrids showed a high rooting rate (from 50 to 73%) during rooting in *in vitro.* However, it is important not only to optimize the rooting of *Juglans* microshoots but also to successfully propagate the maximum number of genotypes available. According to Winkelmann (2012), one option approach to improve rooting could be the application of plant growth promoting rhizobacteria (PGPR) by using some strains that have been shown to produce auxin (IAA) such as *Bacillus* strains which could improve root formation (see Erturk et al. 2010 in case of kiwifruit). Another option is the application of transformants carrying the *rol* (root loci) genes of *Agrobacterium rhizogenes* which were shown to have a better rooting ability in several species (Christensen and Müller, 2009).

The inoculation of the endophytic bacteria strains in *Juglans* could be applied not only in research but also in commercial propagation. More recent studies have focused on the beneficial effects of endophytic bacteria in micropropagation. According to Ulrich et al. (2008), inoculation of poplar tissue culture with *Paenibacillus* sp. led not only to higher numbers but also longer roots in micropropagation. Also, according to an article published recently by Quambusch et al. (2014), the inoculation of the endophytes *Rhodopseudomonas* sp. N-I-2 and *Microbacterium* D-I-1 in difficult-to-propagate *Prunus avium* genotypes led to a positive effect on rooting. According to our findings, this result

requires further analysis. The endophytic bacterium, *Moraxella* sp., which has only been identified in genotypes with good propagation rates (see 2.3) could be inoculated in shoot cultures of other *Juglans* genotypes. These preliminary results could provide useful information for the micropropagation of the *Juglans* species to increase the optimal propagation of the *Juglans* genotypes.

Although there have been a few studies on fungi related to endophytic bacteria in tissue cultures of woody plants, particularly *Juglans* species, Dolcet-Sanjuan (1996) showed that *Juglans regia*, inoculated with the arbuscular mycorrhizal fungi *Glomus mosseae* or *G. intraradices*, significantly improved plant survival when transferred to nursery, nearly 60 and 80% of plants colonized with *Glomus mosseae* or *G. intraradices* survived, respectively, while only 20% of the control continued growth. Bourrain (2009) determined that endomycorrhiza *Glomus* sp., fungi had a positive effect in *Juglans regia*, including faster re-growth and superior survival of *J. regia* under greenhouse conditions. This result could also be applied in the commercial propagation of *J. x intermedia* in the future.

3.4.2 Knowledge transfer to commercialization

The aim of our project was to provide new *Juglans* hybrid genotypes to the market, based on 55 newly established *Juglans* hybrid genotypes from NW-FVA and test their growth behaviour in greenhouses and field trials. However, introduction of new genotypes to the market will comprise five main steps: Selection of promising candidates, production of ramets by micropropagation, clonal field testing, certification and registration of tested clones according to the German Law on Forest Reproductive Material as well as cooperation with commercial micropropagation labs as well as publicity and sales promotion.

This thesis has mainly shed light into first two steps of the introduction, and the results of this thesis could contribute to a cooperation project between NW-FVA and the Institut für Pflanzenkultur (IFP), which is based in Solkau, Schnega. Furthermore, based on the traditional cooperation between NW-FVA and silvaSELECT® (SilvaSELECT Gehölze GmbH is distributing selected, vegetatively reproduced woody plants under license), the high quality of new *Juglans* hybrid genotypes will be selected and distributed under the trademark silvaSELECT®, and soon can start with commercial scale micropropagation.

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Appendices

Awards and honors

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Publications and papers

Pham Ngoc Tuan, Nguyen Van Ket, Nguyen Thanh Sum. (2007*)"Using Plant tissue culture technique for conservation Glyptostropus pensilis (Staunton K.koch).* Journal of Agriculture and Forestry Engineering No: 1 & 2/2007- University of Agriculture and Forestry, Ho Chi Minh City, Viet Nam.

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Oral Presentations

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