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Genetic Formation of Paradox Hybrids (Juglans L.) Revealed by nrDNA IGS8-ETS1 Region

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**Abstract**

Genus *Juglans* (walnuts) is one of the most economically important tree crops for healthy food and wood production. Paradox is a famous rootstock in the walnut industry for a number of important features, including fast growth and resistance to some diseases. Paradox commonly refers to black walnut-Persian walnut hybrid. Nuclear rDNA IGS region, typically bi-parentally inherited, with rapid evolution and broad existence in all eukaryotic genomes, was found to be of importance in revealing genetic background of the walnut hybrids. Both parental genetic components (around 87.50% to 88.89%) and novel genetic components (around 11.11% to 12.50%) were detected in the nrDNA IGS8-ETS1 region of the Paradox genome. The inheritance was commonly one-parent-dominated in each hybridization event. Our results indicated that genetic formation of Paradox hybrids involved in *J. regia* (sect. *Juglans*) and the following 6 black walnut species (sect. *Rhysocaryon*), i.e*.*, *J. hindsii*, *J. californica*, *J. major*, *J. nigra*, *J. microcarpa* and *J. hirsuta*. The nrDNA IGS8- ETS1 region is helpful in understanding the genetic basis of hybrids.

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*Keywords*: *Juglans* L.; Paradox hybrid; genetic formation; nuclear ribosomal DNA; IGS8-ETS1 region

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# Introduction

*Juglans* (Juglandaceae) has about 20 species of 3 sections (sect. *Juglans*, sect. *Rhysocaryon* and sect. *Cardiocaryon*), and is one of the economically important tree crops for nut and wood production in the world [1,2,3]. Nut-producing cultivars of *J. regia* are commonly grafted onto rootstocks. Paradox hybrids have become one of the most important rootstock resources in California walnut industry since the early 1970s because the scion of walnuts grafted on Paradox have increased vigor and resistance to some diseases, e.g., walnut blight (*Xanthomonas campestris* p.v. *juglandis*) and Armillaria root and crown rot (*Armillaria mellea*) [4,5]. The Paradox also presented excellent performances in China in aspects of gardening and agroforestry use [3]. Thus, it has attracted extensive attentions from both walnut industry and academic researchers.

*Juglans* sect. *Rhysocaryon* with 16 black walnut species is unusual in the angiosperms for its intercontinental and insular distribution endemic to and spanning the Americas [6]. This indicates a great potential in association with crop improvement of the Persian walnut (*J. regia* of sect. *Juglans*, commonly used for nut-producing) and high quality wood for cabinetry as well as agroforestry potential for rehabilitating degraded areas such as cloud forest areas [6]. Therefore, an in-depth study on the Paradox would be valuable for walnut breeding and the commercial industry.

Paradox was developed in 1893 by Luther Burbank through artificial hybridization between California walnut and Persian walnut, but at that time he did not recognize northern (*J. hindsii*) and southern (*J. californica*) California black walnuts as distinct species [5,7,8]. Because of Burbank’s breeding work, Paradox primarily refer to the offspring of a northern California black walnut pollinated by a Persian walnut. Now, the name is commonly applied to any black walnut-Persian walnut hybrid (between sect. *Juglans* and sect. *Rhysocaryon*) because of the outstanding performances [5]. Due to gene flow among black walnut species, there exists considerable genetic contribution from species other than *J. hindsii* to the formation of Paradox based on the analysis of the nrDNA ITS sequences [5]. In any case, inconsistency in the rate and direction of concerted evolution will inevitably limit the utility of superimposed nucleotide additive patterns to detect hybrids in all cases [5]. The power of ITS sequences themselves is limited because of the factors such as homogenization via concerted evolution [5, 9].

**Nomenclature**

nrDNA Nuclear ribosomal DNA

ETS external spacer

IGS intergenic spacer region

IGS8-ETS1 region The IGS8 primer anneals ca. 300bp downstream from the 5’ end of the 18S ribosomal

DNA gene [6,10]. The JugETS1 primer anneals the 5’ end of the nrDNA ETS region ITS internal transcribed spacers

cpDNA Chloroplast DNA

NJ tree Neighbor-joining tree

BGI Beijing Genome Institute

Using sequences from five cpDNA non-coding spacer regions (*trn*T-*trn*F, *psb*A-*trn*H, *atp*B-*rbc*L, *trn*V- 16S rRNA and *trn*S-*trn*fM) and three nuclear DNA regions [ETS, ITS and the second intron of the *LEAFY* gene], 14 out of 16 black walnut species were phylogenetically analyzed [6,10]. Molecular data verified the earlier inference by Manning that the black walnut species are quite closely related [6, 10-15].

Species background of Paradox affects the performance of seedlings as rootstocks. Because the nuts from which Paradox seedlings are grown are collected from wild trees, their genetic backgrounds are not generally known [5]. Based on nut morphology and nuclear sequences, some of the Paradox source trees appear to have greater genetic contributions from species other than the ones that their cpDNA sequence profiles matched [5]. Thus, development of more effective markers from the genome to infer the parentage of individual Paradox seedlings is highly desirable [5]. In the previous study, only five black walnut species were sampled to trace the source of Paradox [5]. Therefore, it is necessary to increase sampling size from *Juglans* sect. *Rhysocaryon* to analyze the genetic components of Paradox hybrids.

Possibly ETS can accumulate great diversity in its sequence and could have potential in improving the estimation of phylogenies of different plant groups [6,9,16]. In this study, we report new insights involving genetic formation of Paradox hybrids in *Juglans* based on the sequence data from the nrDNA IGS8-ETS1 region.

# Materials and Methods

* 1. *Sampling*

Four categories of materials were used in this study (Table 1): A Paradox: three Paradox hybrid cultivars, ‘Zhongning Qi’(*J. hindsii* × *J. regia*), ‘Zhongning Qiang’ (*J. major*  *J. regia*) and ‘Zhongning Yi’ (*J. regia*  *J. hindsii*), with fast growth and disease resistance, introduced from University of California after 1999. B Parental materials: *J. hindsii* northern California black walnut, *J. californica* southern California black walnut and *J. major* (Arizona black walnut) of sect. *Rhysocaryon*, and *J. regia* of sect. *Juglans*. *J. regia* was represented by two cultivars (*J. regia* ‘Liaoning 5’ and *J. regia* ‘Xifu 5’) (sequenced in this study) and *J. regia* isolate UC 151 (downloaded sequence from GenBank) [5, 6]. C Two individual plants of *J. sigillata* (the formerly reported species) of sect. *Juglans* (for comparison). D The other three genera of Juglandaceae: *Cyclocarya paliurus* (genus *Cyclocarya* Iljinskaja), *Pterocarya stenoptera* (genus *Peterocarpus* Kunth.), and *Carya cathayensis* and *Carya illinoensis* (genus *Carya* Nattall.) as outgroups of genus *Juglans*. Fresh leaves of the above 14 accessions for sequencing were collected in spring 2009 and dried immediately using silica gel for DNA extraction.

Eighty-two sequences of the 14 accessions were deposited in GenBank. Part of the sequences used in this study were downloaded from GenBank. GenBank accession numbers are shown in Table 1. The source of each sample is indicated using symbol(s) in Table 1 as follows:  Arboretum, Forestry Academy of Yunnan Province, Kunming City, Yunnan Province, China;  Resources Nursery, Forestry Bureau of Luoning County, Henan Province, China;  Beijing Botanical Garden;  see Stone et al. (2009) [6].

* 1. *DNA extraction, PCR amplification and cloned sequencing*

Genomic DNA extraction was conducted following the procedure of Plant Genomic DNA Kit (DP305) from Tiangen Biotech (Beijing) Co., Ltd., China. The primer pair (IGS8 and JugETS1) of the nrDNA IGS8- ETS1 region was reported by Stone et al. (2009) [6]. PCR amplification was conducted following the protocol of TaKaRa Code: DR100B. The PCR program was as follows: preheating at 94°C for 4 min.; 34cycles of 94°C for 1 min., 58°C (annealing temperature) for 40 s and 72°C for 1.4 min.; 8 min at 72°C for final extension. PCR amplification was performed in an Applied Biosystems Veriti™ 96-Well Thermal Cycler (Model#: 9902, made in Singapore). The amplicons were resolved simultaneously on 2% agarose gels (Promega, the USA) run in 1 x TAE buffer at 3 V cm–1 for 3.5 h and were stained with ethidium bromide. Band patterns were documented and photographed with the Gel Documentation System of Transilluminator BINTA2020D (Liaoning Langke Business and Trade Co. Ltd., China). The 100-bp Ladder DNA size marker (100 to 1500bp) was from Tiangen Biotech (Beijing) Co., Ltd., China. PCR products (Fig. 1) were dug out

from the gel using a sterilized scalpel for purification and sent to BGI for cloned sequencing. The fragments were cloned into the pMD18-T Vector (D101A) (TaKaRa Biotechnology (Dalian) Co., Ltd.). Seven to twenty-eight independent clones for each sample were randomly taken and sequenced in both directions using a 3730xl DNA analyzer (Applied Biosystems, Foster City, California, USA) with the M13F(−47) and M13R(−48) primers.

Table 1. GenBank accession numbers of the sequences of each sample used in this study

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| No. | Name of species/variety/cultivar | Isolate No. | Accession No. | Source |
| 1 | *Carya cathayensis*. | H35e5 | JN872758 |  |
| 2 | *Carya illinoensis* | H9e1 | JN872759 |  |
| 3 | *Cyclocarya paliurus* | H67e4 | JN872760 |  |
| 4 | *Pterocarya stenoptera* | H65b6 | JN872761 |  |
| 5 | *Juglans sigillata* | H46b9 | JN872762 |  |
| 6 | *Juglans sigillata* | H47d7 | JN872763 |  |
| 7 | *Juglans regia* ‘Xifu 5’ | H44c7 | JN872764 |  |
| 8 | *Juglans regia* ‘Xifu 5’ | H44d8 | JN872765 |  |
| 9 | *Juglans regia* ‘Liaoning 5’ | HT19a1 | JN872766 |  |
| 10 | *Juglans regia* ‘Liaoning 5’ | HT19e2 | JN872767 |  |
| 11 | *Juglans regia* ‘Liaoning 5’ | HT19f1 | JN872768 |  |
| 12 | *Juglans californica* | H3B1 | JN872769 |  |
| 13 | *Juglans californica* | H3G1 | JN872770 |  |
| 14 | *Juglans californica* | H3DD2 | JN872771 |  |
| 15 | *Juglans californica* | H3d2 | JN872772 |  |
| 16 | *Juglans californica* | H3b10 | JN872773 |  |
| 17 | *Juglans hindsii* | HT76F2 | JN872774 |  |
| 18 | *Juglans hindsii* | HT76a11 | JN872775 |  |
| 19 | *Juglans hindsii* | HT76D1 | JN872776 |  |
| 20 | *Juglans hindsii* | HT76A10 | JN872777 |  |
| 21 | *Juglans hindsii* | HT76D10 | JN872778 |  |
| 22 | *Juglans hindsii* | HT76C5 | JN872779 |  |
| 23 | *Juglans hindsii* | HT76b12 | JN872780 |  |
| 24 | *Juglans hindsii* | HT76d12 | JN872781 |  |
| 25 | *Juglans hindsii* | HT76e11 | JN872782 |  |
| 26 | *Juglans major* | H71d12 | JN872783 |  |
| 27 | *Juglans major* | H71c12 | JN872784 |  |
| 28 | *Juglans major* | H71e12 | JN872785 |  |
| 29 | *Juglans major* | H71g12 | JN872786 |  |
| 30 | *Juglans major* | H71h12 | JN872787 |  |
| 31 | *Juglans major* | H71a12 | JN872788 |  |
| 32 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w141e2 | JN872789 |  |
| 33 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w141d3 | JN872790 |  |
| 34 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w8d2 | JN872791 |  |
| 35 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w141f2f | JN872792 |  |
| 36 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w8d3 | JN872793 |  |
| 37 | ‘Zhongning Qi’i (*Juglans hindsii* × *Juglans regia*) | HT24w8a3 | JN872794 |  |
| 38 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w8a1 | JN872795 |  |
| 39 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w8g2 | JN872796 |  |
| 40 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w8f3 | JN872797 |  |
| 41 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w8f2 | JN872798 |  |
| 42 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w141f3 | JN872799 |  |
| 43 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w141A9 | JN872800 |  |
| 44 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w141B9 | JN872801 |  |
| 45 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w141C9 | JN872802 |  |
| 46 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w141G9 | JN872803 |  |
| 47 | ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w141c2 | JN872804 |  |

|  |  |  |  |
| --- | --- | --- | --- |
| 48 ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w141G4 | JN872805 |  |
| 49 ‘Zhongning Qi’ (*Juglans hindsii* × *Juglans regia*) | HT24w141c3 | JN872806 |  |
| 50 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32d7 | JN872807 |  |
| 51 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32b7 | JN872808 |  |
| 52 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32b9 | JN872809 |  |
| 53 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32b10 | JN872810 |  |
| 54 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32dd8 | JN872811 |  |
| 55 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32e7 | JN872812 |  |
| 56 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32e8 | JN872813 |  |
| 57 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32e9 | JN872814 |  |
| 58 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32e10 | JN872815 |  |
| 59 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32f8 | JN872816 |  |
| 60 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32f9 | JN872817 |  |
| 61 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32g6 | JN872818 |  |
| 62 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32g9 | JN872819 |  |
| 63 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32g7 | JN872820 |  |
| 64 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32d8 | JN872821 |  |
| 65 ‘Zhongning Qiang’ (*Juglans major* × *Juglans regia*) | H32c7 | JN872822 |  |
| 66 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1a1 | JN872823 |  |
| 67 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1a2 | JN872824 |  |
| 68 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1a7 | JN872825 |  |
| 69 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1b1 | JN872826 |  |
| 70 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1b4 | JN872827 |  |
| 71 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1b7 | JN872828 |  |
| 72 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1c2 | JN872829 |  |
| 73 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1c5 | JN872830 |  |
| 74 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1c7 | JN872831 |  |
| 75 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1d2 | JN872832 |  |
| 76 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1d3 | JN872833 |  |
| 77 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1d7 | JN872834 |  |
| 78 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1b6 | JN872835 |  |
| 79 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1e3 | JN872836 |  |
| 80 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1f3 | JN872837 |  |
| 81 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1g4 | JN872838 |  |
| 82 ‘Zhongning Yi’ (*Juglans regia* × *Juglans hindsii*) | H1c6 | JN872839 |  |
| 83 *J. venezuelensis* | 4237 | FJ043007.1 |  |
| 84 *J. venezuelensis* | 4235 | FJ043006.1 |  |
| 85 *J. steyermarkii* | 4368 | FJ043005.1 |  |
| 86 *J. steyermarkii* | 4330 | FJ043004.1 |  |
| 87 *J. regia* | UC151 | FJ043003.1 |  |
| 88 *J. olanchana* | 4363 | FJ043001.1 |  |
| 89 *J. nigra* | 1534 | FJ043000.1 |  |
| 90 *J. nigra* | AR 37 | FJ042999.1 |  |
| 91 *J. neotropica* | 4259 | FJ042998.1 |  |
| 92 *J. mollis* | 4154 | FJ042996.1 |  |
| 93 *J. microcarpa* | 4061 | FJ042995.1 |  |
| 94 *J. mandshurica* | 4062 | FJ042994.1 |  |
| 95 *J. major* | 4387 | FJ042993.1 |  |
| 96 *J. jamaicensis* | 4261 | FJ042992.1 |  |
| 97 *J. jamaicensis* | 4199 | FJ042991.1 |  |
| 98 *J. hirsute* | 4124 | FJ042989.1 |  |
| 99 *J. hindsii* | 4280 | FJ042988.1 |  |
| 100 *J. californica* | 4290 | FJ042985.1 |  |
| 101 *J. boliviana* | 4116 | FJ042984.1 |  |
| 102 *J. australis* | 4394 | FJ042983.1 |  |
| 103 *J. ailanthifolia* |  | FJ042982.1 |  |

Notes: In the isolate no., e.g., “H35” or “HT19” or “H3” or “HT76” is the plant (sample) No. used in the laboratory, “e5” or “a1” or

“DD2” or “A10” is the clone No. during cloned sequencing.

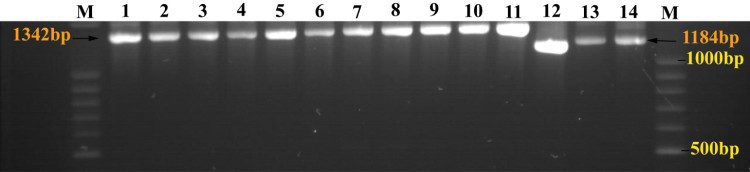


Fig. 1. PCR products of the nrDNA IGS8-ETS1 region of the 14 accessions for cloned sequencing. 1=‘Zhongning Qi’ (*J. hindsii* × *J. regia*), 2=‘Zhongning Qiang’ (*J. major* × *J. regia*), 3=‘Zhongning Yi’ (*J. regia* × *J. hindsii*), 4=*J. hindsii*, 5= *J. major*, 6=*J. californica*, 7=*J. regia* ‘Liaoning 5’, 8=*J. regia* ‘Xifu 5’, 9=individual No.1 of *J. sigillata*, 10=individual No.2 of *J. sigillata*, 11=*Cyclocarya paliurus*, 12=*Pterocarya stenoptera*, 13=*Carya illinoensis*, 14=*Carya cathayensis*. M is the 100-bp Ladder DNA size marker.

* 1. *Data analysis*

Nucleotide sequences were edited and manually corrected by eye using Sequencher (v. 4.6). Alignment was conducted using Clustalx [17]. The fragment of the nrDNA IGS8-ETS1 region was about 1146bp in *Pterocarya stenoptera* (outgroup), 1178bp in *Carya illinoensis* (outgroup), 1184bp in *Carya cathayensis* (outgroup), 1339bp in *Cyclocarya paliurus* (outgroup) and 1342bp to 1345bp among the accessions in sect. *Juglans* (including primer sites). The sequence haplotype diversity of the isolate was analyzed using DnaSP (DNA Sequences Polymorphism version 5.10.01) software [18]. The sequence haplotypes of each accession (excluding outgroups) were retained in the dataset for computation (Table 2 and Fig. 2).

Since sequences of some related species downloaded from GenBank were not long enough, it was decided that the final dataset included 103 sequences/35 accessions in an alignment of 1148bp in length (ranging from position 131 to 1258 from the 5’ end of the nrDNA IGS8-ETS1 region) for formal computation. Totally, 191 parsimony-informative sites were obtained. The NJ tree (Fig. 2) was created using PAUP 4.0b10 [19].

Table 2. The sequence haplotype diversities of the nrDNA IGS8-ETS1 region of the Paradox hybrids and the parental materials based on cloned sequences

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Name of sample | No. of haplotypes (No. of cloned sequences)1 | Haplotype diversity | Unaligned length 2  (Aligned length) (bp) | Number of polymorphic sites 3  (bp) |
| ‘Zhongning Qi’ 4 | 18 (21) | 0.9714 | 1342(1343) | 62 |
| ‘Zhongning Qiang’ 5 | 16 (17) | 0.9926 | 1342(1346) | 57 |
| ‘Zhongning Yi’ 6 | 17(19) | 0.982 | 1342(1345) | 52 |
| *J. hindsii* 7 | 15 (17) | 0.9853 | 1342(1344) | 37 |
| *J. californica* 8 | 20 (28) | 0.9630 | 1342(1342) | 29 |
| *J. major* 9 | 8 (8) | 1.0000 | 1342(1344) | 20 |
| *J. regia* ‘Xifu 5’ 10 | 6 (7) | 0.9524 | 1343(1343) | 11 |
| *J. regia* ‘Liaoning 5’ 11 | 7 (11) | 0.8182 | 1343(1343) | 17 |
| Total | 107 (128) |  |  | 285 |
| Average | 13.375 (16) | 0.9581 | 1342.25 | 35.625 |

Notes: 1 Number of the isolate sequence haplotype with the number of cloned sequence in brackets; 2 Total number of sites excluding sites with gaps/missing data; 3 Number of polymorphic sites within individual (Sites with alignment gaps were not considered); 4 , 5 and 6 Paradox hybrids; 7 , 8 and 9 Parental materials of Paradox in sect. *Rhysocaryon*; 10 and 11 Parental materials of Paradox in *J. regia* of sect. *Juglans*.

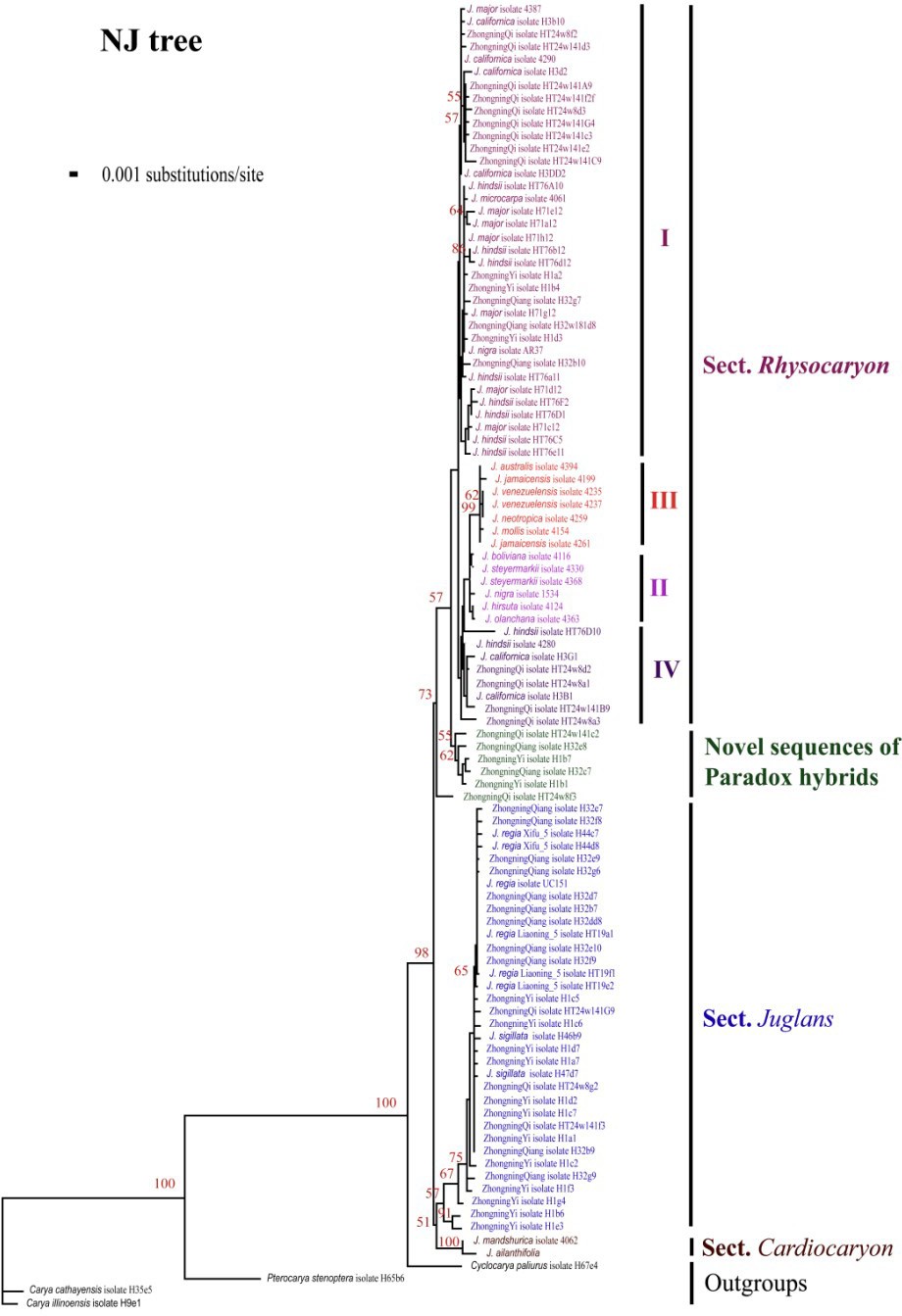


Fig. 2 The NJ strict consensus tree created by PAUP 4.0b10 showing reconstructed phylogeny of the Paradox hybrids in genus *Juglans*, Juglandaceae based on Juckes-Cantor model. The numbers above the branch are bootstrap values (%)/jackknife values (%) of 1 × 103 replicates [Tree length=529, Consistency index (CI)=0.6106, Homoplasy index (HI)=0.3894, Retention index (RI)=0.8601].

# Results

The nrDNA IGS8-ETS1 sequences are typically bi-parentally inherited [5, 6, 9]. As shown in Fig.2, six novel isolates in the Paradox hybrid genomes (i.e., ZhongningQi\_isolate\_ HT24w141c2, ZhongningQi\_ isolate\_HT24w8f3, ZhongningQiang\_isolate\_H32e8, ZhongningQiang\_isolate\_ H32c7, ZhongningYi\_ isolate\_H1b1 and ZhongningYi\_isolate\_H1b7) were isolated, occupying 11.11% of the total number of isolates in ‘ZhongningQi’, 12.5% in ‘Zhongning Qiang’ and 11.76% in ‘Zhongning Yi’.

Three paternal-like isolates (16.67%) (i.e*.*, ZhongningQi\_isolate\_HT24w8g2, ZhongningQi\_isolate\_ HT24w141G9 and ZhongningQi\_isolate\_HT24w141f3) of ‘Zhongning Qi’ were detected and grouped together with *J. regia* in sect. *Juglans* (the male parent) and 13 maternal-like isolates (72.22%) of ‘Zhongning Qi’ were detected and grouped together with *J. hindsii* of sect. *Rhysocaryon* (the female parent). Three maternal-like isolates (18.75%) (i.e., ZhongningQiang\_isolate\_H32b10, ZhongningQiang\_isolate\_ H32g7 and ZhongningQiang\_isolate\_H32w181d8) of ‘Zhongning Qiang’ were detected and grouped together with *J. major* in sect. *Rhysocaryon* (the female parent) and 11 paternal-like isolates (68.75%) of ‘Zhongning Qiang’ were detected and grouped together with *J. regia* in sect. *Juglans* (the male parent). Three paternal-like isolates (17.65%) (i.e., ZhongningYi\_H1a2, ZhongningYi\_isolate\_H1b4 and ZhongningYi\_ H1d3) of ‘Zhongning Yi’ were detected and grouped together with *J. hindsii* in sect. *Rhysocaryon* (the male parent) and 12 maternal-like isolates (70.59%) of ‘Zhongning Yi’ were detected and grouped together with *J. regia* of sect. *Juglans* (the female parent) (Fig. 2).

In brief, three kinds of isolates (maternal-like, paternal-like and novel isolates) from the nrDNA IGS8- ETS1 region of each Paradox hybrid were detected. The parental-like isolates totally covered about 87.5% to 88.89% in the total number of isolates. Genetic contribution to the formation of the Paradox hybrid cultivars was commonly one-parent-dominated in the nrDNA IGS8-ETS1 region in each hybridization event. Novel isolates of each hybrid was around 11.11% to 12.5%. All of the isolates from the Paradox hybrid were grouped within genus *Juglans*. No molecular evidence was detected to show that plants of sect. *Cardiocaryon* have participated in the formation of Paradox hybrids.

# Discussion and conclusion

* 1. *Genetic features of the nrDNA IGS8-ETS1 region*

As a gene family, the sequence of the nrDNA IGS region has a large copy number [9, 16]. Two aspects were found in the bi-parental inheritance process of the nrDNA IGS8-ETS1 region of Paradox hybrids. The first was the addition of genetic components from both parents, which was characterized significantly as one- parent-dominated in each hybridization event. The second was the nucleotide mutations (occurrence of novel isolates) in the nrDNA IGS8-ETS1 region which can be regarded as indicators of heterosis. Paradox hybrids formed a highly heterozygous plant group with a broader genetic basis, suggesting a great value in breeding of excellent rootstocks and high quality wood-producing trees. A quantitative study on gene flow and heterozygosity of black walnut species would improve breeding and utilization of Paradox hybrids [20, 21].

* 1. *Genetic background of Paradox hybrids*

The nrDNA IGS8-ETS1 sequence data did not support the species level treatment of *J. sigillata* Dode. This means that there is a single species (i.e., *J. regia*) in *Juglans* sect *Juglans*. This result was also supported by other studies [10, 22, 23]. Thus, the relationship between the Paradox hybrids and the parent *J. regia* is not questionable because of the monotypic sect. *Juglans*. However, the relationship between Paradox hybrids and the parent of black walnut species from sect. *Rhysocaryon* is comparatively complicated because of the existence of gene flow among black walnut species.

In this study, 14 (87.5%) out of 16 black walnut species were included in our analysis. The other two tropical taxa (*J. pyriformis* and *J. olanchana* var. *standleyi*) were not included, since *J. pyriformis* had no published sequence data available, and the quality of downloaded sequence of *J. olanchana* var. *standleyi* was poor. These two species were planted by the local people in association with coffee fincas (cafetales) for desirable traits like shade, quality wood and edible nuts, or occasionally seen as street trees, throughout Guatemala and other countries including Colima and Mexico (*J. olanchana* var. *standleyi*), and Veracruz and Mexico (*J. pyriformis*), having provided no genetic contribution to the formation of Paradox [6, 24].

In the NJ tree (Fig. 2), four clades (clades I to IV) were recognized in sect. *Rhysocaryon*. Clades I plus IV contained five black walnut species belonging to the temperate group (namely, *J. hindsii*, *J. californica*, *J. major*, *J. nigra* and *J. microcarpa*) with a distribution of northern Mexico, the United States and southern Canada [2, 22, 23]. The fact that a number of parental-like isolates of the Paradox hybrids were grouped closely with the temperate group of black walnut species, indicated that deeper relationships existed between the Paradox hybrid cultivars and the five temperate black walnut species (i.e., *J. californica*, *J. nigra*, *J. microcarpa*, *J. hindsii* and *J. major*)(Fig. 2). In history, the 5 temperate species were used extensively as rootstocks or wood-producing trees, and *J. nigra* was also planted for edible nuts [20, 24]. Two accessions (*J. nigra* 1534 and *J. nigra* AR37) [6] were grouped either closer to the rest 4 temperate black walnut species or closer to a subtropical species *J. hirsuta* (western Mexico distribution), suggesting the existence of gene flow between *J. nigra* and the temperate black walnut species possibly including *J. hirsuta* (Fig. 2). The other two diverged groups (clades II and III) of black walnut species in sect. *Rhysocaryon* have a Mesoamerica and southern America distribution [6, 24]. One group contained *J. boliviana* (Peru), *J. steyermarkii* (Gautemala), *J. olanchana* (Mexico, Guatemala, Honduras and Nicaragua) and *J. hirsuta*, as shown in clade II (Fig. 2). Another group consisted of *J. australis* (Argentina), *J. jamaicensis* (Cuba), *J. venezuelensis* (Venezuela), *J. neotropica* (Venezuela) and *J. mollis* (Mexico) as shown in clade III (Fig. 2). Human activity involving these species was limited. There was no molecular evidence to suggest that the two subtropical/tropical groups have made any genetic contribution to the formation of Paradox hybrids according to our knowledge.

In short, genetic background of Paradox hybrids was affected by *J. regia* and the following 6 black walnut species, i.e*.*, *J. hindsii*, *J. californica*, *J. major*, *J. nigra*, *J. microcarpa* and *J. hirsuta*. Our study indicated that the nrDNA IGS8-ETS1 region is useful in understanding the formation mechanisms and genetic basis of hybrid cultivars.

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# References

1. Lu AM, Stone DE, Grauke LJ. Juglandaceae. In Flora of China, edited by Wu ZY, Raven PH. Sci. Press, Beijing and Mo. Bot. Gard. Press, St. Louis, MO, pp 277–285; 1999.
2. Gunter LE, Kochert G, Giannasi DE. Phylogenetic relationships of the Juglandaceae. Plant Syst. Evol. 1994; 192:11–29.
3. Pei D, Lu XZ. Walnut germplasm resources in China (in Chinese). Beijing: China Forestry Publishing House; 2011.
4. McGranahan GH, Catlin PB. *Juglans* rootstocks. In: Rootstocks for Fruit Crops. edited by Rom RC, Carlson RF, Wiley, New York, pp 411–449, 1987.
5. Potter D, Gao RY, Baggett S, Mckenna JR, McGranahan GH. Defining the sources of Paradox: DNA sequence markers for North American walnut (*Juglans* L.) species and hybrids. Scientia Hort. 2002; 94: 157– 170.
6. Stone DE, Oh SH, Tripp EA, Rios GLE, Manos PS. Natural history, distribution, phylogenetic relationships, and conservation of Central American black walnuts (*Juglans* sect. *Rhysocaryon*). J. Torrey Bot. Soc. 2009; 136:1**–**25.
7. Burbank L.*Luther Burbank: His Methods and Discoveries and their Practical Application*. Vol. II. Luther Burbank Press, New York; 1914.
8. Howard WL. Luther Burbank’s plant contributions. University of California Agricultural Experiment

Station Bulletin, University of California, Berkeley. p.691; 1945.

1. Poczai P, Hyvönen J. Nuclear ribosomal spacer regions in plant phylogenetics**:** problems and prospects. Mol. Biol. Rep. 2010; 37:1897–1912.
2. Aradhya MK, Potter D, Gao FY, Simon CJ. Molecular phylogeny of *Juglans* (Juglandaceae): a biogeographic perspective. Tree Genet. Genom 2007; 3:363–378.
3. Manning WE. The classification within the Juglandaceae. Ann. Mo. Bot. Gard. 1978; 65:1058–1087.
4. Fjellstrom RG, Parfitt DE. Walnut (*Juglans* spp.) genetic diversity determined by restriction fragment length polymorphisms. Genome 1994; 37:690–700
5. Fjellstrom RG, Parfitt DE. Phylogenetic analysis and evolution of the genus *Juglans* (Juglandaceae) as determined from nuclear genome RFLPs. Plant Syst. Evol. 1995; 197:19–32.
6. Stanford AM, Harden R, Parks CR. Phylogeny and biogeography of *Juglans* (Juglandaceae) based on

*mat*K and ITS sequence data. Am. J. Bot. 2000; 87:872**–**882.

1. Manos PS, Stone DE. Evolution, phylogeny and systematics of the Juglandaceae. Ann. Mo. Bot. Gard. 2001; 88: 231–269.
2. Rogers SO, Bendich AJ. Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. Plant Molecular Biology 1987; 9:509-520.
3. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. Bioinformatics 2007; 23:2947–2948.
4. Librado P, Rozas J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data.

Bioinformatics 2009; 25:1451–1452

1. Swofford DL. PAUP\*: Phylogenetic analysis using parsimony (\*and related methods). Version 4.0b10. Sunderland, MA: Sinauer Associates; 2002.
2. Beede RH, Hasey JK. History of the walnut in California, pp. 8–15 in *Walnut Production Manual,* edited by DE Ramos. Division of Agriculture and Natural Resources, Univ. California, Oakland; 1998.
3. Wu YM, Pei D, Xi SK, Li JR. A study on the genetic relationship among species in *Juglans* L. using RAPD markers. Acta Hort. Sinica 2000; 27:17–22.
4. Chen LH, Hu TX, Zhang F. Identification of relationship between *Juglans regia* L. and *J. sigillata* Dode by RAPD markers. J. Sichuan Agri. Univ. 2007; 25:513–516.
5. Stone DE. Biology and evolution of temperate and tropical Juglandaceae. In: *Evolution, systematics and fossil history of the Hamamelidae*. Vol.2, ‘Higher’ Hamamelidae. Systematics Association Special Volume 40B, edited by Crane PR and Blackmore S, Oxford: Clarendon Press; pp 117**–**145; 1989.
6. Whittemore AT, Stone DE. *Juglans*. In: Flora of North America: North of Mexico*.* Vol.3. Manoliophyta, Magnoliidae and Hamamelidae, Flora of North America Editorial Committee. New York: Oxford University Press, pp 425**–**428; 1997.
7. McGranahan GH, Hansen J, Shaw DV. Inter-specific and intra-specific variation in California black walnuts. J. Am. Soc. Hort. Sci. 1988; 113:760–765.