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

Zhili Suo [a], Dong Pei [b, *], Qingguo Ma [b], Xiaobai Jin [c]

[a] *State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, 20 Nan Xin Cun, Haidian district, Beijing 100093, China*

[b] *State Key Laboratory of Tree Genetics and Breeding, Research Institute of Forestry, Chinese Academy of Forestry, Yiheyuan Hou, Beijing 100091, China*

[c] *Beijing Botanical Garden, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China*

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

* Corresponding author. Tel.: +86 10 62889624
E-mail address: peidonggu@163.com

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1. 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.

2. Materials and Methods

2.1. 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].

2.2. 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.

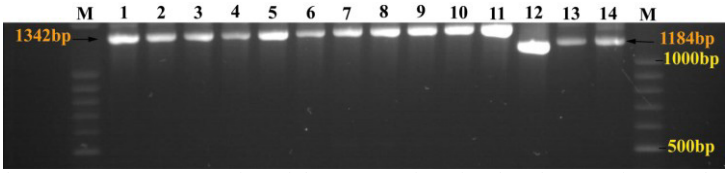

Fig. 1. PCR products of the nrDNA IGS8-ETS1 region of the 14 accessions for cloned sequencing. 1='Zhongning Qi' (*J. hindsii* × *J. regia*), 2='Zhongning Qiang' (*J. major* × *J. regia*), 3='Zhongning Yi' (*J. regia* × *J. hindsii*), 4=*J. hindsii*, 5= *J. major*, 6=*J. californica*, 7=*J. regia* 'Liaoning 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.

2.3. 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*.

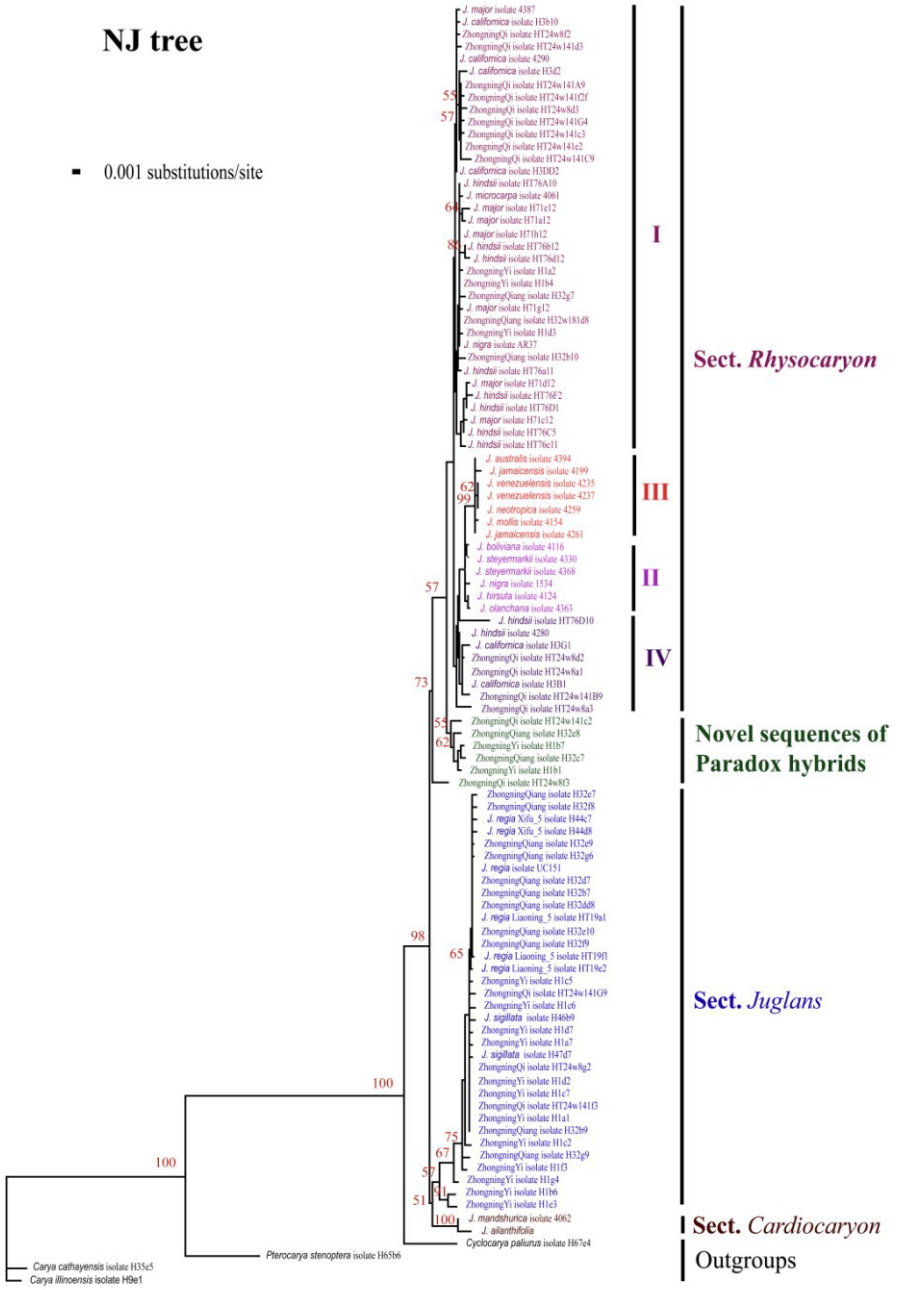

Fig. 2 The NJ strict consensus tree created by PAUP 4.0b10 showing reconstructed phylogeny of the Paradox hybrids in genus *Juglans*, Juglandaceae based on Juckes-Cantor model. The numbers above the branch are bootstrap values (%)/jackknife values (%) of 1×10^3 replicates [Tree length=529, Consistency index (CI)=0.6106, Homoplasy index (HI)=0.3894, Retention index (RI)=0.8601].

3. 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.

4. Discussion and conclusion

4.1. 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].

4.2. 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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