Original article

Analysis of SSR information in the pistil transcriptome and the development and characterization of novel tri-nucleotide SSR markers in *Corylus*

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Summary

Hazelnuts (*Corylus*) are one of the most important nut crops and woody oil plants, having high economic and nutritional value. It is important to study the taxonomy, phylogenetics, and genetic diversity in the genus *Corylus*. In this study, we used the previously constructed pistil transcriptome of the Ping'ou hybrid hazelnut cultivar 'Dawei' to analyze the SSR information and develop novel tri-nucleotide SSR markers in *Corylus*. The results identified 16,339 sequences containing SSRs, resulting in a percentage of 19.72%. An SSR locus was identified for every 5.67 kb of the examined sequences, and 497 motif sequence types were identified. The di-nucleotide repeat type (63.82%) was the most abundant, followed by the tri-nucleotide repeat type (24.54%). (AAG/CTT)_n repeats were dominant among the tri-nucleotide repeats, followed by (AAT/ATT)_n, (ATC/ATG)_n, (AGG/CCT)_n and (ACC/GGT)_n repeats. The sequences with a tri-nucleotide repeat were used to design 160 primer pairs, of which 122 primer pairs showed successful PCR amplification in 'Dawei'. Twenty-two primer pairs that showed polymorphism among the 13 tested samples of the three *Corylus* species were selected, with an average PIC value of 0.50. A dendrogram based on 82 alleles of the 13 samples using the developed SSR primer pairs was generated. The samples were clustered into three main groups in accordance with the species and showed a complete separation in the dendrogram. In conclusion, transcriptome sequencing is an effective tool for SSR marker development, and the newly developed SSR primer pairs will help in genetic and breeding studies in *Corylus* and related species.

Keywords

hazelnut, molecular markers, polymorphism, SSR distribution, tri-nucleotide repeats

Introduction

Hazelnuts (*Corylus*) are one of the most important nut crops and woody oil plants, and have a very high economic and nutritional value. The genus *Corylus* is widely distributed across temperate regions of the northern hemisphere. There have been different opinions regarding the taxonomy of *Corylus*, which is most commonly considered a member of the Birch family, Betulaceae and of the order Fagales

Significance of this study

What is already known on this subject?

- Hazelnuts (*Corylus*) are one of the most important nut crops having high value. It is important to study the taxonomy, phylogenetics and genetic diversity in the genus *Corylus*. Tri-nucleotide repeats were found to be more robust than di-nucleotide repeats when using capillary electrophoresis.

What are the new findings?

- In this study, we used the pistil transcriptome of the Ping'ou hybrid hazelnut cultivar 'Dawei' to analyze the SSR information. SSR locus was identified for every 5.67 kb of the examined sequences in the whole transcriptome database. The di-nucleotide repeat type was the most abundant, followed by the tri-nucleotide repeat type. Twenty-two novel tri-nucleotide SSR markers with high PIC value among three species of *Corylus* were developed.

What is the expected impact on horticulture?

- The results indicating that transcriptome sequencing is an effective tool for the development of SSR markers. The newly developed 22 SSR markers will help in genetic and breeding studies in *Corylus* and related species.

(Mehlenbacher, 2003; Bassil et al., 2005a; Muehlbauer et al., 2014). However, some taxonomists classify it in the subfamily Corylaceae of the family Betulaceae (Chen et al., 1999; Yoo and Wen, 2002; Bassil et al., 2013). The number of species recognized by the taxonomists ranged from 7 to 25, depending on the authority (Mehlenbacher, 2003; Boccacci et al., 2015). Among these, the European hazelnut, *C. avellana* L., is the species of commercial cultivation. In China, there are commonly considered eight hazelnut species and two varieties distributed from 24°31'N to 51°42'N and 85°55'E to 132°12'E. Taxonomists in China also have different opinions in the division of species and varieties native to China, especially the taxonomic status of *C. kweichowensis* Hu or *C. heterophylla* Fisch. var. *sutchuenensis* Franch (Ma et al., 2014). In addition, Ping'ou hybrid hazelnuts (*C. heterophylla* Fisch. × *C. avellana* L.), which are hybrids obtained from Ping hazelnuts from northeast China and European hazelnuts introduced from Italy, have been widely cultivated in twenty-odd provinces of China since 2000 (Liang et al., 2012). Dozens of cultivars and varieties of Ping'ou hybrid hazelnuts are grown

in China, but only sixteen are the main cultivars. The mix of the cultivars/varieties is one of the primary problems in hazelnut production in China. Thus, it is important to study the taxonomy, phylogenetics and genetic diversity in the genus *Corylus*.

Microsatellites or simple sequence repeats (SSRs) are tandem repeats of short DNA sequences extensively distributed throughout the whole genome in both coding and non-coding regions and are considered ideal molecular markers. The distinguishing features of SSR loci are multi-allelic, co-dominant inheritance patterns, reproducibility, high polymorphism, locus specificity, and transferability to related species and genera (Powell et al., 1996). SSRs can be derived from genomic sequences (genomic SSRs) and expressed sequence tags (EST-SSRs). Compared with genomic SSRs, EST-SSRs have several special advantages due to either a relatively higher transferability or a potential gene function (Wei et al., 2011), which have been evaluated in many studies (Poncet et al., 2006; Luro et al., 2008; Wang et al., 2010). At present, more than 230 SSR loci have been developed in *Corylus* (Bassil et al., 2005a, b, 2013; Boccacci et al., 2005; Gürcan and Mehlenbacher, 2010a, b; Gürcan et al., 2010a), which have been widely used in germplasm identification, genetic diversity, linkage map construction and disease-resistant seedling selection (Mehlenbacher et al., 2006; Boccacci et al., 2006, 2013; Gürcan et al., 2010b; Sathuvalli et al., 2011; Bassil et al., 2013; Beltramo et al., 2014; Zong et al., 2015). Most of the above markers were developed from microsatellite-enriched libraries, which are derived from genomic sequences. Only 20 EST-SSRs, characterized in *Corylus*, were derived from an initial whole-transcriptome assembly (Boccacci et al., 2015). The repeat motifs are mainly di-nucleotide repeats, tri-nucleotide repeats and quad-nucleotide repeats possessing a very low ratio. Research showed that di-nucleotide repeats have the disadvantage of high amounts of stuttering, which easily lead to mis-calling, whereas tri-nucleotide repeats and other longer core motifs can significantly reduce arbitrary bindings. Tri-nucleotide repeats were found to be more robust than di-nucleotide repeats when using capillary electrophoresis (Zane et al., 2002; Morgante et al., 2002; Amos et al., 2007; Cipriani et al., 2008; Li et al., 2014).

With the development of next-generation sequencing techniques, transcriptome sequencing offers greater expressed sequence tags, which provide a time- and cost-effective way to develop EST-SSRs, especially for the no-reference genomic species (Mardis, 2008; Morozova and Marra, 2008; Metzker, 2010; Wang et al., 2010; Shi et al., 2011). In our previous study, we performed a pistil transcriptome sequencing during different pollination combinations and stages of Ping'ou hybrid hazelnut cultivars 'Dawei' using Illumina HiSeq 2000. In this study, we analyzed the SSR information of the pistil transcriptome sequencing results and successfully developed 22 tri-nucleotide repeat EST-SSR markers from the sequences with transferability in both *C. heterophylla* and *C. avellana*. This study developed conserved orthologous tri-nucleotide repeat EST-SSR markers for hazelnuts, which will facilitate gene mapping, linkage map development, genetic diversity study and marker-assisted selective breeding for the genus *Corylus*.

Materials and methods

Previous pistil transcriptome sequencing

In our previous study, we performed 7 groups of transcriptome sequencing during different pollination combina-

TABLE 1. Pollination treatment of the pistils of 'Dawei'.

Code	Treatment
CK	Un-pollinated pistils
C0	Compatible pollination, 0 h
C1	Compatible pollination, 1 h
C4	Compatible pollination, 4 h
IC0	Incompatible pollination, 0 h
IC1	Incompatible pollination, 1 h
IC4	Incompatible pollination, 4 h

tions and stages of the pistils of the Ping'ou hybrid hazelnut cultivar 'Dawei' (Table 1). All the pistils used in the 6 treatments were in the full blooming stage. We used the pollen of Ping'ou hybrid hazelnut cultivar 'Liaozhen No. 7' to conduct a compatible pollination, and the pollen of 'Dawei' to conduct an incompatible pollination, based on pollination tests both in the laboratory and in the field. The un-pollinated blooming pistils were used as a control. RNA extraction, library construction, sequencing, *De novo* assembly and sequence annotation were performed by Beijing Genomics Institute (BGI) (Shenzhen, China). Using the Illumina HiSeq™ 2000 platform, the total sequence output was 42,046,035,660 nt; 82,847 Unigenes were assembled and annotated with the total length of 108,232,640 bp (data unpublished).

SSR loci identification and primer design

The MISA program, MISA (http://pgrc.ipk-gatersleben.de/misa/misa.html) was used to identify the SSR loci. The minimum repeat number was set to six for the dinucleotides, five for the tri- and tetra-nucleotides, and four for the penta- and hexa-nucleotides. Primer premier 5.0 (Premier Biosoft International, CA, USA) was used to design the primer pairs flanking the SSR loci with perfect tri-nucleotide motifs using the following parameters: (1) the primers were 12–25 nucleotides long, and the length of the products ranged from 100 to 300 bp; (2) the GC content ranged from 40% to 70%; (3) the T_m value ranged from 55°C to 65°C, and the T_m value of the forward and the reverse primer was no more than 5°C; (4) the primers were devoid of secondary structure or consecutive tracts of a single nucleotide; and (5) the primer designed area was limited to the middle region, with 30 bp removed from the ends of the sequence. In total, 160 pairs of tri-nucleotide repeat SSR primers were randomly selected to perform the following experiment. The primers were synthesized by the Beijing Genomics Institute (BGI, Shenzhen, China).

PCR amplification and fragment analysis

The designed primers were first tested for amplification using Hy1, Ping'ou hybrid hazelnut main cultivar 'Dawei' and for polymorphism and transferability using 12 other samples (Table 2). All samples were obtained from our germplasm repository located in Yanqing, Beijing, China. Samples from Hy2 to Hy5 were the other main cultivars of Ping'ou hybrid hazelnuts. Samples from H1 to H4 were wild Ping hazelnuts collected from Zhalantun, the Inner Mongolia Autonomous Region, Dunhua, Jilin Province, Tieling, Liaoning Province and Weichang, Hebei Province, respectively. Samples from A1 to A4 were European hazelnut cultivars collected from the National Clonal Germplasm Repository (NCGR) in Corvallis, Oregon, USA.

DNA was extracted from 0.2 g of healthy young leaf tissue by using a modified procedure described by Zong et al.

TABLE 5. Polymorphic tri-nucleotide repeats EST-SSRs in *Corylus*.

Code	Primer source	Repeat motif	Forward primer (5'-3')	Reverse primer (5'-3')	Expected size (bp)	Annealing °C	Nr. of alleles	PIC value	Transfer-ability ¹	Nr. of GO annotations ²
CAF-1	Unigene25456_All_11631_3	CAC(3*8)	AAGACTGGAGATTGGATCAGGAG	CAAACTTGCTCAGGAAACTCA	150	57	5	0.62	Hy, H, A	None
CAF-2	Unigene10437_All_10198_1	AAC(3*5)	TTTCGCTTACATATCTCTGAACC	CTAGACTTTTCCCTTACGCGGTCTT	149	56	5	0.62	Hy, H, A	None
CAF-3	Unigene14189_All_10533_4	ATA(3*7)	ACAGAGAGGGTCAATTATTGTCA	GGTAATCACATTTTGGATGTCTCA	119	58	4	0.61	Hy, H, A	None
CAF-4	Unigene26339_All_11736_3	TCA(3*6)	CAATGAGATGCAAGAAATAGAGGCT	TAAATCTGAAACACCCAAAACCT	139	55	5	0.60	Hy, H, A	2
CAF-5	Unigene33161_All_12286_3	CTG(3*7)	CTCTTTGATCATATCCCTCCTCC	AGTTCCAGGTAATCCAGAACTCA	116	56	4	0.60	Hy, A	None
CAF-6	Unigene26183_All_11718_1	TGT(3*6)	ACTCGTTCATGTCTCCTTCAACT	GAAAGTCGACAAACAAAACAAAC	155	58	3	0.58	Hy, H, A	None
CAF-7	Unigene1858_All_8547_1	GAG(3*6)	GAAATCAGCAAAACTACAGGACG	TTTCTTTTCTTCTCCTCCTCTG	132	57	4	0.54	Hy, H	6
CAF-8	Unigene19267_All_10981_3	CGG(3*7)	ATCCTCGAGAGCAGCGAC	AATAATCTTGCCTGCTTTCCG	159	54	4	0.52	Hy, H, A	2
CAF-9	Unigene5476_All_9219_4	AGA(3*6)	CAAAAGAAAAGAAAGAAACAGCCA	TTTATCCCTATATTTTCCCGCG	115	54	4	0.52	Hy, H	1
CAF-10	Unigene1109_All_8480_5	GAT(3*6)	CAGGAGCATGAGGAGTTAGAAGA	CAAAATTCCTCTTTTGCCTCAGAT	115	55	3	0.50	Hy, H, A	None
CAF-11	Unigene6597_All_9656_5	TAT(3*5)	GGAGGTGGTCTAAATTTGGCTA	TTTTGATTACTCTTTTGAIGGCA	155	52	4	0.50	Hy, H, A	7
CAF-12	Unigene41504_All_12815_5	TGG(3*5)	GTGAAGACAAGGAGTTTTCGCTA	TTTATCATTGAGGATGAGAAAG	158	56	3	0.49	Hy, H, A	5
CAF-13	Unigene34497_All_12434_3	TTC(3*6)	CCTTTCTCCCTTTCTTCCCTTA	CAGAAAAAGATCCATTTCAGCTTG	158	55	3	0.48	Hy, H, A	None
CAF-14	Unigene10076_All_10118_5	TGA(3*5)	AGCGAAAAAGTTCTAAGGTGGT	ACTTGTAGGCATGACTTTTGCAT	122	57	4	0.48	Hy, H	None
CAF-15	Unigene45215_All_12848_3	CCT(3*6)	CAGTTGGTTGGATCAGGTGAG	GTACGACTCTCAATGACTGTGCC	136	60	4	0.48	H, A	None
CAF-16	Unigene5498_All_9227_2	TTG(3*6)	AAAAAGACTCAATCTTGGGAAGC	AGAAAGGTGGTGGAAAGAAAAAG	127	55	3	0.47	Hy	4
CAF-17	Unigene25748_All_11676_2	ACC(3*7)	CATCAAGTCAGGAGGAGACACAC	ATGGGTAATGTGAATGGAAGAGA	150	57	3	0.47	Hy, H	4
CAF-18	Unigene27334_All_11801_5	CTC(3*5)	CAAGCACCTCCTGATATTCG	GACAGAGTAACGTGTCTTGGG	139	57	4	0.46	H, A	2
CAF-19	Unigene7477_All_9953_4	ATC(3*7)	ATCAAGCAATGGATCTTCTTCC	GAGAAGACAGATCACTCAAAGGG	159	60	3	0.45	Hy, H, A	None
CAF-20	Unigene29011_All_11890_1	TTC(3*5)	GATCAAGAGCTTCTGGTATGTGG	TGAACACCATCCTTCTCCTTG	136	56	3	0.41	Hy, A	27
CAF-21	Unigene39625_All_12780_3	ACT(3*7)	TCTCCACACTTGATAACCAAAAA	TTAGGGATCTAATGGAGTGCAAA	155	54	4	0.36	Hy, H	5
CAF-22	Unigene21960_All_11258_2	AGG(3*5)	ATGGTGAGGAGGTAGAGATCGTT	TCATCGCTAGAAATCATCATGACC	160	60	3	0.34	Hy, H	9

¹: indicated the different transferability among 3 *Corylus* species, Hy (Ping'ou hybrid hazelnuts, *C. heterophylla* Fisch.), H (Ping hazelnuts, *C. heterophylla* Fisch.) and A (European hazelnuts, *C. avellana* L.).

²: GO annotations were listed in the Supplementary Table S1.

TABLE 2. Plant samples for selected primers.

Code	Sample name	Latin name
Hy1	Dawei	<i>C. heterophylla</i> Fisch. ×
Hy2	Yuzhui	<i>C. avellana</i> L.
Hy3	Liaozhen No. 3	
Hy4	Liaozhen No. 7	
Hy5	Liaozhen No. 8	
H1	Ping-ZLT	<i>C. heterophylla</i> Fisch.
H2	Ping-DH	
H3	Ping-TL	
H4	Ping-WC	
A1	Willamette	<i>C. avellana</i> L.
A2	Hall's Giant	
A3	Tonda Gentile delle Langhe	
A4	Tombul	

(2015). The PCR was performed in a total volume of 20.0 μ L containing 10.0 μ L 2 $\times$ Taq PCR Mastermix (Tiangen Biotech (Beijing) Co., Ltd.), 0.5 mM each primer, and 10 ng genomic DNA. The PCR conditions were as follows: a pre-denaturation step at 94°C for 5 min, then 30 cycles of denaturation 30 s at 94°C, annealing 30 s at the optimal temperature for each primer pair, as reported in Table 5, and extension 30 s at 72°C; the final elongation step was at 72°C for 5 min. The PCR products were separated on 6% denaturing polyacrylamide gels and visualized by silver nitrate staining following the protocol described by Pang et al. (2003).

Data analysis and marker assessment

EmEditor and R software were used to analyze SSR information in the transcriptome data. The distribution of different repeat types, number and frequency of EST-SSRs were calculated. The frequency of the SSRs was computed as $fc(\%) =$

$c/n \times 100$, where c is the number of the SSRs identified in the transcriptome data, and n is the total number of the unigenes assembled and annotated in the transcriptome data.

Bands in the denaturing polyacrylamide gels electrophoresis were scored as their presence (1) or absence (0). Then, the 0, 1 matrix was converted into the genotype format. The diversity level of gene loci was evaluated using the polymorphic information content (PIC). The PIC value was calculated according to the formula:

$$PIC_i = 1 - \sum P_{ij}^2$$

where P_{ij} is the frequency of the j^{th} allele for the i^{th} marker (Smith et al., 1997) using PopGen 32 (Version 1.32) and PIC-Calculator (Version 0.6) software. Cluster analysis was performed with an UPGMA (Unweighted Pair Group Method with Arithmetic mean) algorithm based on an SM (Simple Matching) similarity matrix converted from the SSR polymorphism data of 13 tested samples. A dendrogram was generated by using the NTSYS-pc software package (Version 2.10e).

Results

Identification of SSR loci in the pistil transcriptome of *Corylus*

Using the MISA analysis, 16,339 sequences were identified as containing SSRs from 82,847 unigenes, resulting in a percentage of 19.72%. Overall, 19,097 SSRs were identified in 16,339 sequences. The number of sequences containing more than one SSR locus was 2,277, and the number of SSRs present in compound formation was 1,001. An SSR locus was identified for every 5.67 kb of the examined sequences.

The number of SSRs for di-, tri-, tetra-, penta- and hexa-nucleotide repeat types was 12,188, 4,686, 293, 501 and 428, respectively. A total of 497 motif sequence types

FIGURE 1. Frequency of identified SSR repeat types.

TABLE 3. Distribution of different repeat types in the pistil transcriptome of *Corylus*.

Unit size	Number of SSRs	Percent (%)	Frequency (%)
Di-NR	12,188	63.82	14.71
Tri-NR	4,686	24.54	5.66
Quad-NR	293	1.53	0.35
Penta-NR	501	2.62	0.60
Hexa-NR	428	2.24	0.52
Compound	1,001	5.24	1.21
Total	19,097	100	23.05

NR: nucleotide repeats.

TABLE 4. Type, number and percentage of SSRs in *Corylus*.

Repeat type	Number of repeats								
	4	5	6	7	8	9	10	11	12
Di-NR	0	0	3,200	2,249	2,326	2,224	1,673	498	18
Tri-NR	0	2,342	1,422	790	129	0	2	0	1
Quad-NR	0	236	57	0	0	0	0	0	0
Penta-NR	465	36	0	0	0	0	0	0	0
Hexa-NR	427	1	0	0	0	0	0	0	0
Total	892	2,615	4,679	3,039	2,455	2,224	1,675	498	19
Percentage	4.67	13.69	24.50	15.91	12.86	11.65	8.77	2.61	0.10

NR: nucleotide repeats.

were identified, including 12, 62, 69, 119 and 237 of the di-, tri-, tetra-, penta- and hexa-nucleotide repeat types, respectively (Figure 1 and Tables 3 and 4). Among the perfect SSRs, the di-nucleotide repeat type (63.82%) was the most abundant, followed by the tri-nucleotide repeat type (24.54%). The frequency of the di- and tri-nucleotide repeat types in all the assembled unigenes was 14.71% and 5.66%, respectively. The tetra-, penta- and hexa-nucleotide motifs were comparatively less frequent, and together comprised only 6.39% of the total identified SSRs (Table 3). The repeat times of different SSR motifs were distributed from 4 to 12, among which 5 to 9 repeats were the most abundant, with a percentage of 78.61% among the identified SSRs. For *Corylus*, the majority of tri-nucleotide repeats ranged from 15 to 24 bp in length (Table 4).

Figure 1 shows the frequency of the identified SSR repeat types. The di-nucleotide repeats (AG/CT)_n were predominant, representing 46.35% of all of the characterized motifs, followed by the (AT/AT)_n repeats (12.93%) and (AC/GT)_n repeats (4.50%). For the tri-nucleotide repeats, (AAG/CTT)_n repeats were dominant, representing 8.43% of all of the characterized motifs, followed by the (AAT/ATT)_n repeats (3.93%), (ATC/ATG)_n repeats (2.74%), (AGG/CCT)_n repeats (2.56%) and (ACC/GGT)_n repeats (2.45%). The di-nucleotide repeats (CG/CG)_n were the fewest, representing 0.04% of all of the characterized motifs. These above results indicate that transcriptome sequencing is a useful tool for SSR identification in *Corylus*.

Development of novel tri-nucleotide SSR markers in *Corylus* and polymorphism testing

A total of 160 primer pairs were specially designed from the sequences with tri-nucleotide repeat types, which were tested for amplification in the Ping'ou hybrid hazelnut 'Dawei'. Of these primer pairs, 122 amplified repeatedly and reliably, while the other primer pairs produced too many undesirable bands or no bands and were excluded from further analysis. The percentage of the amplification efficiency of the

primers was 76.25%. To validate the level of polymorphism for the newly designed SSR markers and to investigate the transferability, all of the efficient primer pairs were tested on 13 samples of 3 different species of *Corylus* (Table 2). Electrophoresis on 6% denatured polyacrylamide gels showed that 22 primer pairs generated perfect polymorphic PCR products among the 13 samples. Characterization data for the 22 polymorphic loci is summarized in Table 5. The ratio of the polymorphic amplification of the primers was 13.75%. The PIC value ranged from 0.34 for CAF-22 to 0.62 for CAF-1 and CAF-2, with the average value of 0.50. The PIC values for CAF-1 to CAF-11 were higher than 0.50, which were highly informative. The number of alleles ranged from 3 to 5 per locus (mean 3.75) among the 13 samples. Among these primers, CAF-1, CAF-2, CAF-3, CAF-4, CAF-6, CAF-8, CAF-10, CAF-11, CAF-12, CAF-13 and CAF-19 showed transferability in Ping'ou hybrid hazelnuts (*C. heterophylla* Fisch. × *C. avellana* L.), Ping hazelnuts (*C. heterophylla* Fisch.) and European hazelnuts (*C. avellana* L.), with a percentage of 55.00% among the polymorphic primers. CAF-7, CAF-9, CAF-14, CAF-17, CAF-21 and CAF-22 showed transferability in Ping'ou hybrid hazelnuts and Ping hazelnuts. CAF-5 and CAF-20 showed transferability in Ping'ou hybrid hazelnuts and European hazelnuts. CAF-15 and CAF-18 showed transferability in Ping hazelnuts and European hazelnuts. However, primer CAF-16 showed polymorphic amplification only in Ping'ou hybrid hazelnuts. Figure 2 shows 4 groups of PCR products of polymorphism tri-nucleotide repeat EST-SSRs. The data above indicate that the 22 novel tri-nucleotide EST-SSR markers were reasonably polymorphic in *Corylus* and might be useful for other genetic studies.

The application of the developed tri-nucleotide repeats SSR markers

We observed 82 alleles in the 13 samples using 22 developed polymorphic SSR primer pairs. A dendrogram depicting the genetic relations among the 13 samples from three species of *Corylus* was constructed. They were clustered

FIGURE 2. PCR products of polymorphism tri-nucleotide repeat EST-SSRs.

into 3 main groups that were in accordance with the species (Figure 3). Group I contained 5 main cultivars of the Ping'ou hybrid hazelnut. It was subdivided into 4 subgroups. 'Dawei' and 'Yuzhui' were included in one subgroup. 'Liaozhen No. 7', 'Liaozhen No. 3' and 'Liaozhen No. 8' were subdivided as single subgroups. Group II contained the 4 varieties of wild Ping hazelnuts, which included 2 subgroups: one comprised 2 varieties, which were collected from Zhalantun, the Inner Mongolia Autonomous Region and Dunhua, Jilin Province, and the other comprised 2 varieties collected from Tieling, Liaoning Province and Weichang, Hebei Province. Finally, group III included 4 cultivars of European hazelnuts, collected from the NCGR in Corvallis, Oregon, USA. It was subdivided into 3 subgroups: the Italian progeny 'Willamette' and 'Tombul' and one of the main cultivars from Turkey were included in one subgroup. 'Tonda Gentile delle Langhe', one of the main cultivars from Italy, and 'Hall's Giant', which originated in Germany, were subdivided as the other two single subgroups.

Discussion

Frequency and distribution of SSRs in *Corylus* pistil transcriptome

Polymorphic SSR markers are important tools for research in genetic diversity, relatedness, evolution, linkage mapping, comparative genomics and gene-based association studies. Deep transcriptome sequencing provides a good resource for the development of SSRs because of the enormous amount of sequence data. Moreover, since the transcriptome is the expressed portion of the genome, markers developed from it should be in or very near the functional regions. Just as the recent reported SSR markers from transcriptome sequences of European hazelnut, 109 polymorphic SSR markers were developed from bark, catkin, leaf and the young seedling transcriptome sequences (Colburn et al., 2017). The SSR markers derived from the pistil transcriptome would increase the likelihood of developing useful markers that are

FIGURE 3. UPGMA dendrogram of 13 tested samples based on alleles at 22 developed SSR loci.

closely linked to functional genes, especially during the pollen-stigma recognition.

In this study, 16,339 sequences were identified containing SSRs from 82,847 unigenes (the total size was 108,232,640), resulting in a percentage of 19.72%. Moreover, the SSR locus was identified for every 5.67 kb of the examined sequences. The frequency of SSRs from the assembled unigenes and the distribution density of SSRs differ greatly between the taxa in the previous studies. The SSR frequency (19.72%) in this study was higher than 14.4% in species of *Amorphophallus* (Araceae) (Zheng et al., 2013), 16.67% in tea (*Camellia sinensis*) (Tan et al., 2013), 17.21% (SSRs ≥ 15 bp) in sesame (*Sesamum indicum* L.) (Zhang et al., 2012), and slightly lower than 23.79% in radishes (*Raphanus sativus* L.) (Wang et al., 2012). For the distribution density, the results of this study were lower than those of the previous study in radishes (one per 3.45 kb) (Wang et al., 2012), tea (*Camellia sinensis* L., one per 3.55 kb) (Sharma et al., 2013), species of *Amorphophallus* (one per 3.63 kb) (Zheng et al., 2013), and Chinese jujube (*Ziziphus jujuba* Mill., one per 3.87 kb) (Li et al., 2014). However, the distribution density of SSRs in *Corylus* was higher than that in the previous study in sesame (one per 6.55 kb for 15 bp and one per 10.81 kb for 18 bp) (Zhang et al., 2012), Chinese medicinal plant (*Epimedium sagittatum* (Sieb. et Zucc.) Maxim, one per 6.69 kb) (Zeng et al., 2010), and Chinese Cymbidium (*Cymbidium ensifolium*) floral transcriptome (one per 17.56 kb) (Li et al., 2013). The frequency of SSRs in various plant genomes was influenced by database-mining tools, the data set size, and the search parameters used to search the SSRs, such as the repeat length threshold and the number of repeat motifs unit (Varshney et al., 2005; Wei et al., 2008). The results showed that the SSR resources in the *Corylus* transcriptome were at the middle level among many other species, and the number of SSRs was sufficient for identifying EST-SSR markers.

As for the repeat types of the SSRs, di-nucleotide repeat motifs were the most frequent repeat type, representing 63.82% of the SSR loci identified, followed by tri-nucleotide repeats (24.54%), and the higher nucleotide repeat motifs, which were less frequent. This is consistent with the EST-SSRs distributions reported in sesame (Zhang et al., 2012), tea (Tan et al., 2013), species of *Amorphophallus* (Zheng et al., 2013), and Chinese Cymbidium floral transcriptome (Li et al., 2013). However, tri-nucleotide repeats were predominant in radish species (Shirasawa et al., 2011; Wang et al., 2012). As the similar research work in Betulaceae, the data of di-nucleotide repeats and tri-nucleotide repeats were 63.9% and 33.8%, respectively, reported by Boccacci et al. (2015) when studying on the Betulaceae EST sequences. While Colburn et al. (2017) reported of the total number of repeats mined from the four hazelnut transcriptomes, 52% were di-nucleotide repeats, 45% tri-nucleotide repeats, while the higher repeat motifs process lower frequencies. Trinucleotide repeats are predominant compared with other motifs in coding sequences. This high frequency is likely due to a selective disadvantage of non-trimeric SSR variants in coding regions and the resulting frameshift mutations (Metzgar et al., 2000).

The most abundant di- and tri-nucleotide repeat motifs were AG/CT (46.35%) and AGG/CCT (8.43%), respectively, in our transcriptome sequences. The same results were also found in other plant species (Wei et al., 2008; Wang et al., 2012; Zhang et al., 2012; Zheng et al., 2013; Tan et al., 2013). In the research of Boccacci et al. (2015), the most common motif of the di-nucleotide repeats was AG/CT (51.1%) and the tri-motifs was AAG/CTT (13.2). The most common SSR

motifs in the hazelnut transcriptome, Colburn et al. (2017) reported, were AG (35.8%) and AAG (12.7%). The CG/GC SSRs were the least abundant motifs in this study, accounting for 0.04% of the total SSRs. A low frequency of CG/GC repeats was also observed in the ESTs of other plants (Wei et al., 2011; Wang et al., 2012; Ueno et al., 2012; Tan et al., 2013) and GC/GC repeats were also not found in some species (Kantety et al., 2002; Liang et al., 2009; Zeng et al., 2010; Zhang et al., 2012).

Polymorphism and genera transferability of the developed SSR markers

In this study, a high success ratio of amplification efficiency (76.25%) was obtained compared with similar studies, and this ratio was similar to that observed for trinucleotide repeats in tea (*Camellia sinensis* L., 72%, Sharma et al., 2013) and Chinese jujube (71.6%, Li et al., 2014). All of the novel SSR primers showed good transferability to other cultivars of the Ping'ou hybrid hazelnut (*C. heterophylla* Fisch. $\times$ *C. avellana* L.) and even to the closely related species Ping hazelnut (*C. heterophylla* Fisch.) and European hazelnut (*C. avellana* L.). Of these markers, 22 generated perfect polymorphic PCR products, with a percentage of 13.75%. Among these, 20 markers showed polymorphism in the Ping'ou hybrid hazelnut (Hy, in Table 5), 19 markers showed polymorphism in the Ping hazelnut (H, in Table 5) and 15 markers in the European hazelnut (A, in Table 5). Generally, due to the conserved nature of EST-SSR, fewer polymorphisms were detected for EST-SSRs than for the genomic SSRs (Eujayl et al., 2004). However, a high polymorphic rate was also observed in some of the EST-SSR studies (Zheng et al., 2013; Tan et al., 2013; Li et al., 2014). The ratio of marker polymorphisms varies among previous studies. For example, 76.3% was observed in the Chinese jujube (Li et al., 2014), 47.5% in Lentils (*Lens culinaris* Medik.) (Kaur et al., 2011), and 12.9% was detected in pigeon peas (*Cajanus cajan* (L.) Millspaugh) (Dutta et al., 2011). The polymorphism ratio mainly depends on the tested samples and the selection criteria. In similar studies in peanut (*Arachis hypogaea* L.), 26 (10.3%) EST-SSRs exhibited polymorphisms between 22 cultivated peanut accessions and 221 (88%) were polymorphic between 16 wild peanut species (Liang et al., 2009); in sesame, 32 (11.59%) genic-SSR markers were polymorphic in 24 cultivars, 18 (6.52%) were polymorphic in one mapping population, and 167 (60.51%) were polymorphic between the 24 accessions and a wild accession (Zhang et al., 2012). The use of more sensitive techniques for DNA fragment size analysis, e.g., capillary electrophoresis, may be expected to show a higher rate of polymorphism.

In this study, only tri-nucleotide repeats were adopted for developing the SSR markers. Tri-nucleotide repeats and other longer core motifs could be more integrated and could significantly reduce arbitrary binning decisions than di-nucleotide repeats (Cipriani et al., 2008). Capillary electrophoresis showed that the tri-nucleotide repeats were more robust than the di-nucleotide repeats (Amos et al., 2007; Cipriani et al., 2008; Li et al., 2014). Tri-nucleotide core repeats are less frequent in most plant genomes (Zane et al., 2002; Morgante et al., 2002); therefore, the development of tri-nucleotide repeats was more convenient when taking advantage of the high-throughput transcriptome sequencing. Genetic markers are based on particularly expressed sequences; thus, they are potentially tightly linked with functional genes that may control certain important phenotypic characteristics (Ramchiary et al., 2011). The results of many similar stud-

ies indicate that large numbers of polymorphic SSR markers can be obtained when large volumes of transcript sequences are used. Compared with other SSR detection methods, the *de novo* RNA sequencing approach was well suited for developing large numbers of genic-SSRs, which could be useful for studying functional diversity, executing marker assisted gene selection and performing association mapping studies in *Corylus*.

The application of the developed tri-nucleotide repeats SSR markers in three *Corylus* species

A total of 82 alleles were detected, using 22 developed polymorphic SSR primer pairs. The PIC value ranged from 0.34 to 0.62, with an average of 0.50. Eleven loci were equal to or higher than 0.50, ranged from 0.50 to 0.62, which were highly informative. In the similar research on hazelnut, 20 polymorphic SSR loci were amplified in 18 samples, the PIC ranged from 0.14 to 0.81, and its mean value was 0.50 (Bocacci et al., 2015). The level of polymorphism was slightly higher than in this study. On the contrary, in both the two studies, the SSR primer pairs revealed less polymorphism comparing with gSSRs used in hazelnut germplasm characterization. Bocacci et al. (2005) developed 18 gSSR and showed a high level of polymorphism in 20 accessions, with the PIC value of 0.64. Bassil et al. (2005a) analyzed 25 gSSR loci in 20 genotypes and the PIC were 0.64. The lower polymorphism of EST-SSRs is usually explained by the location of the genic SSRs in transcribed and hence conserved regions of the genome (Bocacci et al., 2015).

The dendrogram generated from UPGMA cluster analysis was clustered into three main groups related to the species (Figure 3). The hybrids of the Ping'ou hybrid hazelnuts (*C. heterophylla* Fisch. × *C. avellana* L.) were made from 1980 to 1985 by W.J.L. and resulted in a population of more than 2,000 seedlings (Liang et al., 2012). The cultivars were released from 2004 to recent years. The female parents consisted of five advanced selections from wild Ping hazelnuts (*C. heterophylla* Fisch.) from northeast China. The male parents were the mixed pollen collected from 10 seedlings of the European hazelnuts introduced from Italy. Therefore, the genetic relationship of the Ping'ou hybrid hazelnuts was very complex. It was difficult to detect the parentage relationship of these hybrids, but the mix of the cultivars/varieties is one of the problems in hazelnut production in China. In this study, we developed 20 polymorphic SSR primer pairs for Ping'ou hybrid hazelnuts, and they could successfully distinguish among the five tested cultivars.

The Ping hazelnut is one of the wild species native to China and is widely distributed in the northeast part of China. The propagation pattern of the Ping hazelnut, both by sexual propagation and by clonal propagation, leads to the high genetic diversity of this species. The 4 tested samples used in the present study were collected from Zhalantun (121°53'E; 48°08'N), the Inner Mongolia Autonomous Region, Dunhua (128°23'E; 43°36'N), Jilin Province, Tieling (124°17'E; 42°44'N), Liaoning Province and Weichang (117°23'E; 42°08'N), and Hebei Province. We developed 19 polymorphic SSR primer pairs for Ping hazelnuts, and the clustering result is in accordance with the geographical distance.

As for the four tested samples of European hazelnuts, 'Willamette' was the Italian progeny (Mehlenbacher et al., 1991), 'Hall's Giant' originated in Germany (Mehlenbacher, 1991), 'Tonda Gentile delle Langhe' was one of the main cultivars from Italy, and 'Tombul' was one of the main cultivars from Turkey. We developed 15 polymorphic SSR primer pairs

for *C. avellana* L. The clustering result in the present study was similar to the result reported from another related article (Bocacci et al., 2006). The above data indicate that our 22 SSR markers were reasonably polymorphic in the three species of hazelnuts and might be useful for germplasm characterization and genetic mapping.

Conclusions

In this study, we used the previously constructed pistil transcriptome to analyze the SSR information and develop novel tri-nucleotide SSR markers in *Corylus*. We identified 16,339 sequences containing SSRs, resulting in a percentage of 19.72% in the whole database. SSR locus was identified for every 5.67 kb of the examined sequences. SSR information, such as the distribution of different nucleotide repeat types, the type, number and percentage of SSRs and the frequency of identified SSR repeat types, were analyzed in detail. Furthermore, 22 genic SSR primer pairs that could generate perfect polymorphic PCR products among the 13 samples were developed, with an average PIC value of 0.50. A dendrogram based on 82 alleles of the 13 samples using the developed SSR markers was generated. The samples were clustered into three main groups in accordance with the species and showed complete separation in the dendrogram.

In summation, the results in this study further highlight the efficient identification of SSRs from the pistil transcriptome of *Corylus*, indicating that transcriptome sequencing is an effective tool for the development of SSR markers. The developed SSR markers will help in genetic and breeding studies in *Corylus* and related species.

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Competing interests

The authors have declared that no competing interests exist.

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SUPPLEMENTARY TABLE S1. GO Annotations.

Gene ID	GO-Biological process	GO-Molecular function	GO-Cellular component
CAF-1 Unigene25456_All_11631_3	-	-	-
CAF-2 Unigene10437_All_10198_1	-	-	-
CAF-3 Unigene14189_All_10533_4	-	-	-
CAF-4 Unigene26339_All_11736_3	2	GO:0046872/metal ion binding; GO:0016491/oxidoreductase activity	-
CAF-5 Unigene33161_All_12286_3	-	-	-
CAF-6 Unigene26183_All_11718_1	-	-	-
CAF-7 Unigene1858_All_8547_1	6	GO:0004528/phosphodiesterase I activity	GO:0005773/vacuole; GO:0005634/nucleus
CAF-8 Unigene19267_All_10981_3	2	GO:0003723/RNA binding	GO:0005634/nucleus
CAF-9 Unigene5476_All_9219_4	1	-	-
CAF-10 Unigene1109_All_8480_5	-	-	-
CAF-11 Unigene6597_All_9656_5	7	-	GO:0005794/Golgi apparatus; GO:0016023/cytoplasmic membrane-bounded vesicle; GO:0005886/plasma membrane; GO:0016021/integral to membrane
CAF-12 Unigene41504_All_12815_5	5	GO:0010296/prenylcysteine methylesterase activity	GO:0000139/Golgi membrane; GO:0005789/endoplasmic reticulum membrane; GO:0005576/extracellular region
CAF-13 Unigene34497_All_12434_3	-	-	-
CAF-14 Unigene10076_All_10118_5	-	-	-
CAF-15 Unigene45215_All_12848_3	-	-	-
CAF-16 Unigene5498_All_9227_2	4	GO:0016597/amino acid binding	GO:0009570/chloroplast stroma; GO:0009535/chloroplast thylakoid membrane
CAF-17 Unigene25748_All_11676_2	4	GO:0004679/AMP-activated protein kinase activity; GO:0005515/protein binding	GO:0005737/cytoplasm
CAF-18 Unigene27334_All_11801_5	2	-	GO:0016021/integral to membrane
CAF-19 Unigene7477_All_9953_4	-	-	-

SUPPLEMENTARY TABLE S1. Continued.

Gene ID	GO-Biological process	GO-Molecular function	Go-Cellular component
CAF-20 Unigene29011_All_11890_1	27 GO:0007000/nucleolus organization; GO:0009786/regulation of asymmetric cell division; GO:0050826/response to freezing; GO:0000911/cytokinesis by cell plate formation; GO:0010311/lateral root formation; GO:0048829/root cap development; GO:0048825/cotyledon development; GO:0009640/photomorphogenesis; GO:0019915/lipid storage; GO:0010182/sugar mediated signaling pathway; GO:0009933/meristem structural organization; GO:0006468/protein phosphorylation; GO:0016567/protein ubiquitination; GO:0009845/seed germination; GO:0010228/vegetative to reproductive phase transition of meristem; GO:0009737/response to abscisic acid stimulus; GO:0009909/regulation of flower development; GO:0000280/nuclear division; GO:0010162/seed dormancy process	GO:0019199/transmembrane receptor protein kinase activity; GO:0004674/protein serine/threonine kinase activity; GO:0042803/protein homodimerization activity; GO:0005524/ATP binding	GO:0032585/multivesicular body membrane; GO:0016021/integral to membrane; GO:0009986/cell surface; GO:0030139/endocytic vesicle; GO:0005886/plasma membrane
CAF-21 Unigene39625_All_12780_3	5 GO:0033345/asparagine catabolic process via L-aspartate; GO:0006516/glycoprotein catabolic process; GO:0051604/protein maturation	GO:0004067/asparaginase activity	GO:0005829/cytosol
CAF-22 Unigene21960_All_11258_2	9 GO:0048438/floral whorl development; GO:0006261/DNA-dependent DNA replication; GO:0006290/pyrimidine dimer repair; GO:0048449/floral organ formation; GO:0051052; GO:0007049/cell cycle	GO:0005515/protein binding; GO:0003684/damaged DNA binding; GO:0030983/mismatched DNA binding	GO:0005634/nucleus