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ORIGINAL ARTICLE

Study on chloroplast DNA diversity of cultivated and wild pears (*Pyrus* L.) in Northern China

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Abstract Eight pairs of chloroplast DNA (cpDNA) universal primers selected from 34 pairs were used to assess the genetic diversity of 132 pear accessions in Northern China. Among them, six amplified cpDNA fragments showed genetic diversity. A total of 24 variable sites, including 1 singleton variable site and 23 parsimony informative sites, as well as 21 insertion-deletion fragments, were obtained from the combined cpDNA sequences (5309–5535 bp). Two *trn*L-*trn*F-487 haplotypes, five *trn*L-*trn*F-413 haplotypes, five *rbc*L haplotypes, six *trn*S-*psb*C haplotypes, eight *acc*D-*psa*I haplotypes and 12 *rps*16-*trn*Q haplotypes were identified among the individuals. Twenty-one haplotypes were identified based on the combined fragments. The values of nucleotide diversity (P_i), average number of nucleotide differences (k) and haplotype diversity (H_d) were 0.00070, 3.56408 and 0.7960, respectively. No statistical significance was detected in Tajima's D test. Remarkably, the important cpDNA haplotypes and their representing accessions were identified clearly in this study. H_19 was considered as one of the ancient haplotypes and was a divergent centre. H_16 was the most common haplotype of the wild accessions. H_2 was the haplotype representing the most pear germplasm resources (46 cultivars and two wild Ussurian Pear accessions), followed by haplotype H_5 (30 cultivars, two wild Ussurian Pear accessions and four sand pears in outgroups) representing the cultivars 'Dangshan Suli' and 'Yali', which harbour the largest and the second largest cultivation areas in China. More importantly, this study demonstrated, for the first time, the supposed evolution routes of *Pyrus* based on cpDNA divergence in the background of pear phylogeny in Northern China.

Keywords *Pyrus* L. · Chloroplast DNA · Haplotype diversity · Northern China

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Introduction

According to paleontological data, genus *Pyrus* L. of subfamily Pomoideae of family Rosaceae is believed to be of Tertiary or possibly even more ancient origin (Rubtsov 1944). It developed from 22 recognized primary species (Bell et al. 1996) into various species during a long history of cultivation by humans. In China, pear trees originated in the mountainous regions of southwestern China and spread westward and eastward. As one of the three most diverse cultivated pear centres (Vavilov 1951), China has more than 2000 pear germplasm resources safely preserved in the five national fruit germplasm repositories located in Liaoning, Jilin, Xinjiang, Hubei and Yunnan (Cao 2014). Among them, 13 species originated in China, including species with commercial cultivars such as Chinese White Pear *(P. bretschneideri* Rehd.), Chinese Sand Pear (*P. pyrifolia* (Burm.f.) Nakai), Sinkiang Pear (*P. sinkiangensis* Yü) and Ussurian Pear (*P. ussuriensis* Maxim.) (Pu and Wang 1963; Yu 1979). Many of these

varieties have adapted to different environmental conditions and mature in different periods in China.

Provinces and cities in Northern China, including Beijing, Tianjin, Inner Mongolia, Xinjiang, Gansu, Ningxia, Shanxi, Shaanxi, Qinghai, Shandong, Henan, Hebei, Anhui as well as parts of Jiangsu, Liaoning, Jilin and Heilongjiang are rich in pear germplasm resources. These regions are suitable for cultivating the main cultivars of many pear varieties such as 'Yali', 'Xuehuali' and 'Dangshan Suli' of White Pear varieties and 'Nanguoli', 'Jianbali' and 'Huagai' of Ussurian Pear varieties. Moreover, many improved varieties such as 'Cuiguan', 'Huangguan' and 'Zaosu' as well as some varieties introduced from Japan, Korea and Europe are grown in these regions.

Molecular data have been widely applied in studies on genetic diversity and phylogeography of plant species to further understand their evolutionary processes (Montanari et al. 2013; Wuyun et al. 2013; Zong et al. 2014). As a complement to nuclear DNA and a maternally inherited biomarker, chloroplast DNA (cpDNA) has been proved to be a useful and powerful tool in population genetics and phytogeography (Liu 2006; Liu et al. 2012, 2013; Zong et al. 2014) because of its features of uniparental inheritance, nearly neutral evolution, low evolutionary rate and little or no recombination (Clegg and Zurawski 1992). In addition, unlike the nuclear genome, cpDNA can also be used to analyse genetic structure and evolutionary events (Petit et al. 1993; Katayama et al. 2012; Wuyun et al. 2013).

Research on genetic diversity in *Pyrus* has mainly focused on the identification and characterization of cultivars or species using different molecular markers such as random amplified polymorphic DNA (RAPD) (Teng et al. 2001, 2002), amplified fragment length polymorphism (AFLP) (Bao et al. 2008), restriction fragment length polymorphism (RFLP) (Iketani et al. 1998), simple sequence repeats (SSR) (Yamamoto et al. 2001, 2002a, 2002b; Bao et al. 2007; Cao et al. 2007; Katayama et al. 2007; Yao et al. 2010; Sehic et al. 2012; Urrestarazu et al. 2015) and non-coding regions of cpDNA (Kimura et al. 2003; Liu et al. 2012; Wuyun et al. 2013). There has been significant progress in studies on the cpDNA diversity of Chinese pears (Liu 2006; Liu et al. 2012, 2013; Wuyun et al. 2013). However, the genus *Pyrus* shows extremely low chloroplast genome diversity compared with other angiosperms (Katayama and Uematsu 2003). The conservative evolution of cpDNA is valuable for exploring the phylogenetic relationships at many taxonomic levels (Palmer et al. 1985). Despite chloroplast genome conservation, structural alterations such as inversions, translocations, deletions (gaps) and insertions found in hypervariable regions of cpDNA (for example, *acc*D-*psa*I and *rps*16-*trn*Q regions) evolved at a faster rate than other regions. Moreover, these structural alterations could be used for phylogenetic analyses of *Pyrus* species (Liu et al. 2013; Katayama et al. 2012) and reconstructing plant phylogeny at higher taxonomic levels (Downie and Palmer 1992; Doyle et al. 1992; Katayama and Ogihara 1996). Specifically, two large deletions of the non-coding *acc*D-*psa*I and *rps*16-*trn*Q regions in two hypervariable regions have been used to classify cpDNA into three important types: type A has no large deletions, type B contains a 229-bp deletion in the region of *acc*D-*psa*I and type C possesses a 141-bp deletion in the region of *rps*16-*trn*Q. Katayama et al. (2012) identified 25 cpDNA haplotypes based on 36 mutations in the fragments of *acc*D-*psa*I and *rps*16-*trn*Q from 21 *Pyrus* species originating from Asia, Europe and Africa, and they established a Median-joining network based on these 25 cpDNA haplotypes. Wuyun et al. (2013) identified 30 cpDNA haplotypes based on 32 mutations from the same two hypervariable regions of 186 wild pear accessions and generated a haplotype network to illustrate their genetic relationships. The two hypervariable regions containing two large deletions have been proven useful and applicable for the evaluation of genetic diversity or genetic relationships among accessions in *Pyrus*.

Reports on pear cpDNA diversity have focused on the local species in one province or area (Liu et al. 2012; Chang et al. 2014; Zong et al. 2014; Zhang et al. 2016). There have been few reports on cpDNA diversity of different *Pyrus* species in Northern China. Moreover, the phylogenetic relationships among the accessions in Northern China are not clear. Thus, the aim of the current research was to study the diversity of cpDNA of pear accessions in Northern China and explore the evolution routes of *Pyrus* based on cpDNA haplotypes.

Materials and methods

Plant materials

A total of 132 pear accessions were analysed in this study, including 31 Chinese *P. ussuriensis* cultivars, 12 *P. ussuriensis* wild accessions, 56 *P. bretschneideri* cultivars, 16 *P. pyrifolia* cultivars, nine *P. sinkiangensis* cultivars, two *P. xerophila* cultivars and six *P. betuleafolia* wild accessions. In addition, four *P. pyrifolia* cultivars ('Choujuurou' and 'Housui' both from Japan, 'Jiangwan Tangli' from Jiangxi and 'Xiaomeili' from Zhejiang), three *P. communis* cultivars ('Bartlett' and 'Conference' both from England and 'Clapp's Favourite' from America) and two *Malus domestica* accessions ('Ralls' and '*Malus baccata* (L.) Borkh' both from China) were used as outgroups (Table 1).

The 132 pear accessions originated from 16 provinces, including Xinjiang, Liaoning, Jilin, Heilongjiang, Henan, Hebei, Shandong, Qinghai, Gansu, Anhui, Yunnan, Guizhou, Sichuan, Jiangsu, Shanxi and Shaanxi (Fig. 2), and all were preserved in the Chinese National Pear Germplasm Repository in Research Institute of Pomology, Chinese Academy of Agricultural Sciences (Xingcheng, Liaoning), located from 40° 16′ N 120° 06′ E to 40° 50′ N 120° 50′ E. Young and healthy leaves of different accessions were

Table 1 Pear accessions used in this study and haplotype distributions

Species	Code	Accession name	Origin	Haplotypes
P. ussuriensis Maxim.	1	Anli	Hebei	H_8
	2	Anshan No.1	Anshan, Liaoning	H_2
	3	Baihuaguan	Funing, Hebei	H_10
	4	Daxiangshui	Anshan, Liaoning	H_5
	5	Guanhongxiao	Beizhen, Liaoning	H_2
	6	Huagai	Liaoning	H_8
	7	Huangjin Duima	Beizhen, Liaoning	H_2
	8	Huangshanli	Qinglong, Hebei	H_8
	9	Huishanbai	Shenyang, Liaoning	H_5
	10	Jianba	Kaiyuan, Liaoning	H_2
	11	Liuyuexian	Anshan, Liaoning	H_9
	12	Maili	Qinghai	H_7
	13	Manyuanxiang	Suizhong, Liaoning	H_2
	14	Miansuan	Qinglong, Hebei	H_8
	15	Muli	Jianchang, Liaoning	H_2
	16	Nanguoli	Anshan, Liaoning	H_2
	17	Pingli	Liaoning	H_2
	18	Qingtang	Beizhen, Liaoning	H_2
	19	Qiuzi	Beizhen, Liaoning	H_2
	20	Reli	Hebei	H_8
	21	Reqiuzi	Huludao, Liaoning	H_2
	22	Ruan'er	Longzhong, Gansu	H_2
	23	Shatangli	Zhuanghe, Liaoning	H_2
	24	Tianqiuzi	Beizhen, Liaoning	H_2
	25	Xiaohebai	Kaiyuan, Liaoning	H_5
	26	Xiaoxiangshui	Liaoning	H_8
	27	Xiuyanci	Xiuyan, Liaoning	H_5
	28	Yanbian Longjing	Yanbian, Jilin	H_8
	29	Yanbian Xiehuatian	Yanbian, Jilin	H_8
	30	Zisheng 17	Beizhen, Liaoning	H_8
	31	Zaomi	Jianping, Liaoning	H_2
	32	*P. ussuriensis* 'Antu Shanli'	Antu, Jilin	H_12
	33	*P. ussuriensis* 'Duoci Shanli'	Mudanjiang, Heilongjiang	H_2
	34	*P. ussuriensis* 'Hedixiangli'	Linfen, Shanxi	H_16
	35	*P. ussuriensis* 'Kuandian Shanli'	Kuandian, Jilin	H_2
	36	*P. ussuriensis* 'Ning'an Shanli'	Ning'an, Heilongjiang	H_12
	37	*P. ussuriensis* 'Ping'an Suanli 1'	Zhangjiachuan, Gansu	H_16
	38	*P. ussuriensis* 'Ping'an Suanli 3'	Zhangjiachuan, Gansu	H_7
	39	*P. ussuriensis* 'Shanli 24'	Chengde, Hebei	H_5
	40	*P. ussuriensis* 'Suiling Shanli'	Suiling, Heilongjiang	H_5
	41	*P. ussuriensis* 'Wenquan Suanli '	Wushan, Gansu	H_16
	42	*P. ussuriensis* 'Wuci Shanli'	Mudanjiang, Heilongjiang	H_12
	43	*P. ussuriensis* 'Xilin Shanli'	Xilin, Heilongjiang	H_12
P. bretschneideri Rehd.	44	Baipiao	Changli, Hebei	H_5
	45	Baizhi Muyang	Xinglong, Hebei	H_5
	46	Banjinsu	Jinzhou, Liaoning	H_5
	47	Bingtang	Minhe, Qinghai	H_3
	48	Boli	Funing, Hebei	H_5
	49	Boshanchi	Boshan, Shandong	H_2
	50	Changba	Huangxian, Shandong	H_2
	51	Da'aoao	Qingdao, Shandong	H_2
	52	Dadongguo	Lanzhou, Gansu	H_3
	53	Dahebai	Jianchang, Liaoning	H_2
	54	Dayan	Meixian, Shaanxi	H_4
	55	Dangshan Suli	Dangshan, Anhui	H_5
	56	Donghuang	Suiding, Xinjiang	H_2
	57	Ehuang	Dangshan, Anhui	H_5
	58	Etouli	Jianchang, Liaoning	H_2
	59	Fengxian Jitui	Fengxian, Shaanxi	H_3
	60	Fojianxi	Zunhua, Hebei	H_5
	61	Fuli	Zanhuang, Hebei	H_2
	62	Haicheng Cili	Haicheng, Liaoning	H_5
	63	Haitangsu	Siyang, Jiangsu	H_2
	64	Hongsumei	Minhe,Qinghai	H_3
	65	Hongzhi Muyang	Xinglong, Hebei	H_5

Table 1 (continued)

Species	Code	Accession name	Origin	Haplotypes
	66	Huangxian Qiuli	Huangxian, Shandong	H_21
	67	Jidanguan	Qinglong, Hebei	H_2
	68	Jinan Xiaobai	Changqing, Shandong	H_5
	69	Jinan Xiaohuangli	Jinan, Shandong	H_5
	70	Jinchuizi	Zhuanghe, Liaoning	H_5
	71	Jinhua	Jinchuan, Sichuan	H_2
	72	Jinli	Wanrong, Shanxi	H_5
	73	Jingchuan	Jingchuan, Gansu	H_3
	74	Lanzhou Dongguo	Longzhong, Gansu	H_3
	75	Lianyungang Huangli	Lianyungang, Jiangsu	H_5
	76	Liuban	Jianchang, Liaoning	H_2
	77	Liuleng	Xiongyue, Liaoning	H_2
	78	Longdengzao	Jinchuan, Sichuan	H_2
	79	Matihuang	Dangshan, Anhui	H_5
	80	Pingguoli	Yanbian, Jilin	H_3
	81	Qixia Daxiangshui	Qixia, Shandong	H_5
	82	Qixia Xiaoxiangshui	Qixia, Shandong	H_20
	83	Qingpicao	Dangshan, Anhui	H_5
	84	Ruanba	Jinzhou, Liaoning	H_2
	85	Sajin	Meixian, Shaanxi	H_5
	86	Shimen Shuidonggua	Jinchuan, Sichuan	H_5
	87	Suizhong Xiehuatian	Suizhong, Liaoning	H_11
	88	Taihuang	Jiaohe, Hebei	H_5
	89	Xiali	Yuanping, Shanxi	H_2
	90	Xiangchun	Dali, Shaanxi	H_5
	91	Xiangya	Dingxian, Hebei	H_5
	92	Xinxiang	Jinchuan, Sichuan	H_3
	93	Xuehua	Dingxian, Hebei	H_5
	94	Yali	Hebei	H_5
	95	Yangbaixiao	Haicheng, Liaoning	H_2
	96	Yanghongxiao	Jianchang, Liaoning	H_5
	97	Youhongxiao	Jianchang, Liaoning	H_5
	98	Yuanba	Jinzhou, Liaoning	H_2
	99	Zhengzhou Eli	Zhengzhou, Henan	H_3
P. pyrifolia (Burm.f.) Nakai	100	Anhui Xueli	Huizhou, Anhui	H_14
	101	Baozhuli	Chenggong, Yunnan	H_2
	102	Cangxi Xueli	Cangxi, Sichuan	H_2
	103	Dazisu	Daming, Hebei	H_2
	104	Huobali	Jinning, Yunnan	H_3
	105	Jiangwan Tangli[a]	Wuyuan, Jiangxi	H_5
	106	Jinsutang	Qinglong, Hebei	H_2
	107	Lusha	Yuanyang, Yunnan	H_2
	108	Mopan	Yanbian, Jilin	H_2
	109	Tiepi	Dangshan, Anhui	H_2
	110	Xiangmian	Dangshan, Anhui	H_2
	111	Xiaojin	Yan'an, Shaanxi	H_2
	112	Xiaomeili[a]	Xinchang, Zhejiang	H_5
	113	Xingyi Haizili	Xingyi, Guizhou	H_2
	114	Yanbian Dahuang	Yanbian, Jilin	H_2
	115	Yanbian Dashan	Yanbian, Jilin	H_3
	116	Yanbian Mingyue	Yanbian, Jilin	H_2
	117	Yuanxiang	Beidaihe, Hebei	H_3
	118	Choujuurou[a]	Japan	H_5
	119	Housui[a]	Japan	H_5
P. sinkiangensis Yü	120	Guide Changba	Qinghai	H_6
	121	Hongnahe	Tulufan, Xinjiang	H_13
	122	Korla Pear	Korla, Xinjiang	H_2
	123	Kuike Juju	Kuche, Xinjiang	H_1
	124	Lanzhou Changba	Lanzhou, Gansu	H_9
	125	Seer Kefu	Yili, Xinjiang	H_1
	126	Wowo	Qinghai	H_6
	127	Wuwei Xiangjiao	Wuwei, Gansu	H_9
	128	Youjiaotuan	Linxia, Gansu	H_13
P. xerophila Yü	129	Muli	Gansu	H_15
	130	Shageda	Qinghai	H_2

Table 1 (continued)

Species	Code	Accession name	Origin	Haplotypes
P. betuleafolia Bge.	131	*P. betuleafolia* 'Chemingyu Shanli 4'	Puxian, Shanxi	H_8
	132	*P. betuleafolia* 'Hedixiang Duli 1'	Linfen, Shanxi	H_17
	133	*P. betuleafolia* 'Maodushu 1'	Changli, Hebei	H_8
	134	*P. betuleafolia* 'Shanxi Duli 1'	Qinyuan, Shanxi	H_19
	135	*P. betuleafolia* 'Tailinxiang Duli1'	Puxian, Shanxi	H_18
	136	*P. betuleafolia* 'Yipingyuanxiang Duli 2'	Linfen, Shanxi	H_16
P. communis L.	137	Bartlett[a]	England	H_14
(Outgroups)	138	Clapp's Favourite[a]	America	H_14
	139	Conference[a]	England	H_22
Malus domestica	140	*Malus baccata* (L.) Borkh[a]	China	H_23
(Outgroups)	141	Ralls[a]	China	H_24

[a] Outgroup accessions used in this study

collected from trees 10 m apart from each other in the spring of 2014 and maintained in silica gel until use.

DNA extraction and quality of determination

Genomic DNAs were extracted with a modified cetyl trimethyl ammonium bromide (CTAB) method as described by Doyle and Doyle (1987) and subjected to 1.2% agarose gel electrophoresis for quality examination. All good quality DNA samples were stored at −70 °C and adjusted to 10–30 ng μl^{-1} before use.

CpDNA universal primer pairs for PCR amplification

Thirty-four pairs of cpDNA universal primers (Supplementary Table S1) used to explore cpDNA diversity of pear accessions were previously reported (Taberlet et al. 1991; Demesure et al. 1995; Kelchner and Clark 1997; Dumolin-Lapegue et al. 1997; Small et al. 1998; Parducci and Szmidt 1999; Parani et al. 2000; Katayama et al. 2012) and synthesized by Sangon (Shanghai, China). Six random cultivars were selected for PCR amplification to select cpDNA universal primer pairs suitable for subsequent experiments. After PCR amplification, 5 μl of each PCR product was electrophoresed on a 2% agarose gel.

Amplification and sequencing of cpDNA fragments

PCR amplification was carried out in a 40 μl reaction containing 40 ng of DNA template, 0.4 μM of each primer, 200 μM of each dNTP (Tiangen, Beijing, China), 2 mM $MgCl_2$ and 2 U rTaq DNA polymerase (Tiangen, Beijing, China) at the following cycling conditions: 90 °C for 3 min followed by 35 cycles of 90 °C for 30 s, 52 °C for 40 s and 72 °C for 90 s and a final extension at 72 °C for 10 min. PCR amplification conditions for primers cp11 were modified as 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 56 °C for 40 s and 72 °C for 90 s and a final extension at 72 °C for 6 min.

PCR products were electrophoresed, purified from agarose gels and analysed directly by an ABI 3730 sequencer system (Applied Bio systems, Inc., USA). The amplified fragment size was calculated based on an internal DNA standard with Gene Mapper 4.0 software (Applied Bio systems, Inc., USA).

Chloroplast haplotype analyses

Chloroplast DNA regions were aligned using software Clustal X ver2.1 (http://www.clustal.org/download/current/) and then analysed by MEGA ver6.06 (http://www.me-gasoftware.net/index.php). All sequences were saved in both FASTA and MEGA formats for further analyses after being refined manually. Chloroplast DNA fragments from the regions of *trn*L-*trn*F, *trn*L-*trn*F, *rbc*L, *trn*S-*psb*C, *acc*D-*psa*I and *rps*16-*trn*Q were combined by using software PAUP beta ver4.0 (Swofford 2002) (http://www.sciencesoftware.com.cn/search/search_soft_detail12.a-sp.?id=752) for further analysis.

Haplotype number (h), haplotype diversity (H_d), variance of haplotype diversity (V_h), standard deviation of haplotype diversity (S_h), nucleotide diversity (P_i) (Nei and Li 1979), average number of nucleotide differences (k), variable (polymorphic) sites (V_s), singleton variable sites (S_s), parsimony informative sites (P_s) and insertion-deletion fragments (I_g) were calculated based on each cpDNA region and combined regions by using software DnaSP ver5.10.01 (Librado and Rozas 2009).

Tajima's test

Tajima's D values were calculated by using software DnaSP ver5.10.01 (Librado and Rozas 2009). A positive Tajima's D signifies low levels of both low and high frequency polymorphisms, indicating a decrease in balancing selection. A negative Tajima's D signifies an excess of low frequency

polymorphisms relative to expectation, indicating purifying selection (Tajima 1989).

Construction of a haplotype network

The median-joining network was constructed with software Network ver4.6.13 (http://www.fluxus-engineering.com/) based on the cpDNA haplotypes derived from combined regions.

In addition, a complementary approach for reconstruction of a phylogenetic tree based on the plastid data was performed using software TCS ver1.21 (Clement et al. 2000).

Results

Six universal primer pairs for PCR amplification of cpDNA regions

Electrophoresis analysis showed that six cpDNA universal primer pairs, P02 (*trn*L-*trn*F), P03 (*trn*L-*trn*F), P09 (*trn*S-psbC), cp03 (*rbc*L), cp11 (*acc*D-*psa*I) and cp19 (*rps*16-*trn*Q) produced clear, stable and single bands with mutation sites existing in each region.

Genetic diversity and chloroplast haplotypes based on each region and the combined regions and Tajima's test

The *trn*L-*trn*F intergenic region was amplified by primer pairs P02, P03, P14 and cp13, all of which produced clear, stable and single bands. However, mutations were only detected in the sequences amplified by the former two primers. The fragments amplified by primer pairs P02 and P03 were 487 and 403–413 bp, respectively. The *rbc*L and *trn*S-*psb*C cpDNA fragments were aligned into 1270 and 1517 bp, respectively. The lengths of *acc*D-*psa*I and *rps*16-*trn*Q ranged from 725 to 982 bp and 719 to 930 bp, respectively. The length of the combined fragments ranged from 5309 to 5535 bp after alignment.

The polymorphic information based on cpDNA fragments was analysed and is depicted in Table 2. We found one singleton variable site, 23 parsimony variable sites and 21 insertion-deletion fragments in the combined cpDNA regions. Moreover, we observed three parsimony informative sites in *trn*L-*trn*F-487, four parsimony informative sites in *trn*L-*trn*F-413, five parsimony informative sites in *rbc*L, one singleton variable site and four parsimony variable sites in *trn*S-*psb*C, two parsimony variable sites in *acc*D-*psa*I and one parsimony variable site in *rps*16-*trn*Q. Among the six cpDNA regions, *trn*L-*trn*F-413 had two insertion-deletion gaps with lengths of 8 and 10 bp (Table S2); *acc*D-*psa*I had six insertion-deletion gaps with lengths of 1, 2, 5, 10, 22 and 229 bp (Table S4), and *rps*16-*trn*Q had 13 insertion-deletion gaps with lengths of 1, 1, 2, 2, 5, 7, 8, 11, 17, 23, 24, 24 and 141 bp (Table S5). No gaps were found in the *rbc*L and *trn*S-*psb*C regions (Table S3).

Five parsimony informative sites were found in the 229-bp insertion sequence of *acc*D-*psa*I (Table S4) and two were found in the 141-bp insertion sequence of *rps*16-*trn*Q (Table S5). Moreover, the non-coding region *trn*L-*trn*F-413 showed the highest nucleotide diversity ($P_i = 0.00233$) and an average number of nucleotide differences ($k = 0.91869$) (Table 2), while the non-coding region *rps*16-*trn*Q showed the lowest nucleotide diversity ($P_i = 0.00006$) and an average number of nucleotide differences ($k = 0.04476$).

Two *trn*L-*trn*F-487 haplotypes, five *trn*L-*trn*F-413 haplotypes, five *rbc*L haplotypes, six *trn*S-*psb*C haplotypes, eight *acc*D-*psa*I haplotypes and 12 *rps*16-*trn*Q haplotypes were identified among individuals (Table 3). As one of the hypervariable regions of pear cpDNA, intergenic region *rps*16-*trn*Q had the most haplotypes and the highest haplotype diversity ($h = 12$, $H_d = 0.7739$, Table 3), followed in turn by another hypervariable cpDNA region *acc*D-*psa*I ($h = 8$, $H_d = 0.7604$, Table 3) and the intergenic region *trn*L-*trn*F-413 ($H_d = 0.7061$). The *trn*L-*trn*F-487 region had the fewest haplotypes and the lowest haplotype diversity ($h = 2$, $H_d = 0.1411$,

Table 2 Polymorphic information obtained using DnaSP software based on cpDNA fragments of 132 pear accessions

Region	Length range (bp)	Variable sites (V_s)	Singleton variable sites (S_s)	Parsimony informative sites (P_s)	Insertion-deletion fragments (I_g)	Nucleotide diversity (P_i)	Average number of nucleotide differences (k)
*trn*L-*trn*F-487	487	3	0	3	0	0.00087	0.42332
*trn*L-*trn*F-413	403–413	4	0	4	2	0.00233	0.91869
*rbc*L	1270	5	0	5	0	0.00046	0.58420
*trn*S-*psb*C	1517	5	1	4	0	0.00055	0.83669
*acc*D-*psa*I	725–982	5	0	5	6	0.00086	0.61531
*rps*16-*trn*Q	719–930	2	0	2	13	0.00006	0.04476
Combined	5309–5535	24	1	23	21	0.00070	3.56408

Table 3 Chloroplast DNA haplotypes and the diversity and Tajima's *D* identified by using DnaSP

Region	Number of haplotypes (h)	Haplotype (gene) diversity (Hd)	Variance of haplotype diversity (Vh)	Standard deviation of haplotype diversity (Sh)	Tajima's *D*	Significance
trnL-trnF-487	2	0.1411	0.00153	0.039	−0.39349	$P > 0.10$
trnL-trnF-413	5	0.7061	0.00124	0.035	0.47936	$P > 0.10$
rbcL	5	0.1454	0.00174	0.042	−0.73835	$P > 0.10$
trnS-psbC	6	0.6378	0.00054	0.023	−0.17714	$P > 0.10$
accD-psaI	8	0.7604	0.00055	0.024	0.99192	$P > 0.10$
rps16-trnQ	12	0.7739	0.00061	0.025	−0.82144	$P > 0.10$
Combined	21	0.7960	0.00054	0.023	−0.28066	$P > 0.10$

Table 3). The *trn*S-*psb*C and *rbc*L regions showed the lowest (V_h = 0.00054, S_h = 0.023, Table 3) and the highest (V_h = 0.00174, S_h = 0.043, Table 3) variance and standard deviation of haplotype diversity, respectively.

As shown in Table 3, the Tajima's *D* value was positive only in the *trn*L-*trn*F-413 and *acc*D-*psa*I regions and showed no significant differences among the six cpDNA regions ($P > 0.10$).

CpDNA haplotypes characterized by mutations in six regions

Twenty-four haplotypes (21 haplotypes for pear accessions and three for outgroups) were identified among the individuals analysed in this study based on base change characters and gaps, of which two haplotypes, H_23 and H_24, belonged to *Malus* outgroup accessions and H_22 belonged to *P. communis* 'Conference' (Table 1). Haplotypes H_1 to H_22 were found in 2, 48, 12, 1, 36, 2, 2, 11, 3, 1, 1, 4, 2, 3, 1, 4, 1, 1, 1, 1, 1 and 1 pear accessions, respectively. Fig. 1 shows the type and number of haplotypes in each species. Five haplotypes were found in 12 wild *P. ussuriensis* accessions (66.7% in 18 wild pear accessions, 16.7% in H_2 and H_5, 8.3% in H_7, 33.3% in H_12, 25.0% in H_16), six wild *P. betuleafolia* accessions (33.3% in 18 wild pear accessions, 33.3% in H_8, 16.7% in H_16, H_17, H_18 and H_19) and in nine *P. sinkiangensis* cultivars (7.9% in 114 cultivars, 22.2% in H_1, H_6, H_9 and H_13, and 11.1% in H_2). Six

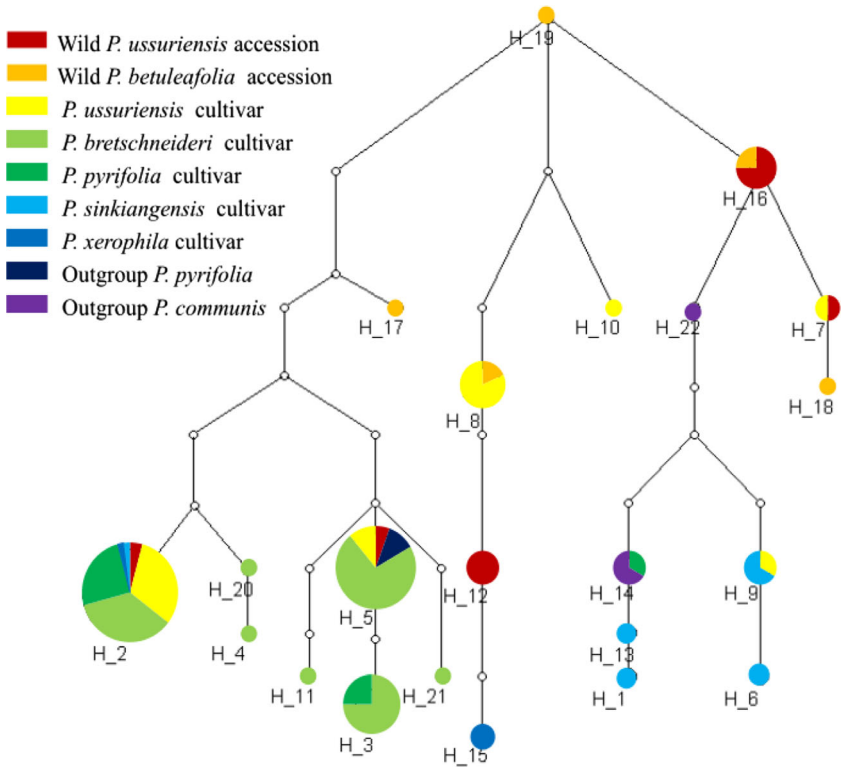

Fig. 1 Genetic relationships of 132 pear accessions and seven pear outgroups based on chloroplast DNA analyses. The accessions of each species are indicated using the same colour-code. Circle size is proportional to the number of individuals per haplotype. Each haplotype is labelled below the *circle*. Gaps are treated as the fifth state

haplotypes were detected in 31 *P. ussuriensis* cultivars (27.2% in 114 cultivars, 48.4% in H_2, 12.9% in H_5, 29.0% in H_8, 3.2% in H_7, H_9 and H_10). Seven haplotypes were identified in 56 *P. bretschneieri* cultivars (49.1% in 114 cultivars, 30.3% in H_2, 16.1% in H_3, 1.8% in H_4, H_11, H_20 and H_21, and 46.4% in H_5). Three haplotypes were detected in 16 *P. pyrifolia* cultivars (14.0% in 114 cultivars, 75.0% in H_2, 18.8 in H_3 and 6.2% in H_14). Two haplotypes were observed in two *P. xerophila* cultivars (1.8% in 114 cultivars, 50.0% in H_2 and H_15) and three *P. communis* cultivars (66.7% in H_14, and 33.3% in H_22). The four *P. pyrifolia* cultivars from China used as outgroup shared the same haplotype H_5.

Figure 1 also shows the species each haplotype contains. H_2 was composed of five pear species, including two wild *P. ussuriensis* accessions, 15 *P. ussuriensis* cultivars, 17 *P. bretschneideri* cultivars, 12 *P. pyrifolia* cultivars, one *P. sinkiangensis* cultivar and one *P. xerophila* cultivar and, therefore, was considered to be one of the main haplotypes in Chinese pear cultivars. H_5 was composed of three pear species, including two wild *P. ussuriensis* accessions, four *P. ussuriensis* cultivars, 26 *P. bretschneideri* cultivars and four *P. pyrifolia* cultivars. Importantly, cultivars 'Dangshan Suli' and 'Yali' from *P. bretschneideri* with the largest and second largest cultivation areas in China belonged to this haplotype.

Geographic distribution of cpDNA haplotype polymorphisms

Twenty-one haplotypes were recognized from 132 pear accessions originating from 16 provinces (Table 1 and Fig. 2). Haplotypes H_1, H_4, H_6, H_10, H_11 and H_15 were only dispersed in pear accessions originating from Xinjiang, Shaanxi, Qinghai, Hebei, Liaoning and Gansu, respectively. The haplotypes H_17, H_18 and H_19 were only dispersed in *P. betuleafolia* originating from Shanxi. Haplotypes H_20 and H_21 were dispersed only in *P. bretschneideri* from Shandong. H_2, the most common haplotype, was dispersed in most pear germplasm resources from 15 provinces, except Henan.

Haplotype network analysis

The median-joining network depicting the relationships among 24 cpDNA haplotypes (21 haplotypes for pear accessions and three for outgroups) derived from the comparison of the cpDNA sequences in the six regions was composed of six parts in different colours corresponding to circle C-1 through circle C-6 (Fig. 3). All haplotypes of pear accessions (H_1 to H_22) could be classified into three types: type A had no large deletion, type B had a 229-bp deletion in the region of *acc*D-*psa*I and type C had a 141-bp deletion in the region of *rps*16-*trn*Q.

H_19 was unique to wild pear accession *P. betuleafolia* 'Shanxi Duli 1' and lay in the torso of the Median-joining network connecting directly or indirectly to the other haplotypes in 6 circles with different colours (Fig. 3). Circle C-2 contained four haplotypes including H_3, H_5, H_11 and H_21. All of them were type B haplotypes with a 229-bp deletion in *acc*D-*psa*I and mainly represented Chinese White Pear cultivars. The circle C-3 contained six haplotypes. Among them, H_1, H_6, H_9 and H_13 were type C

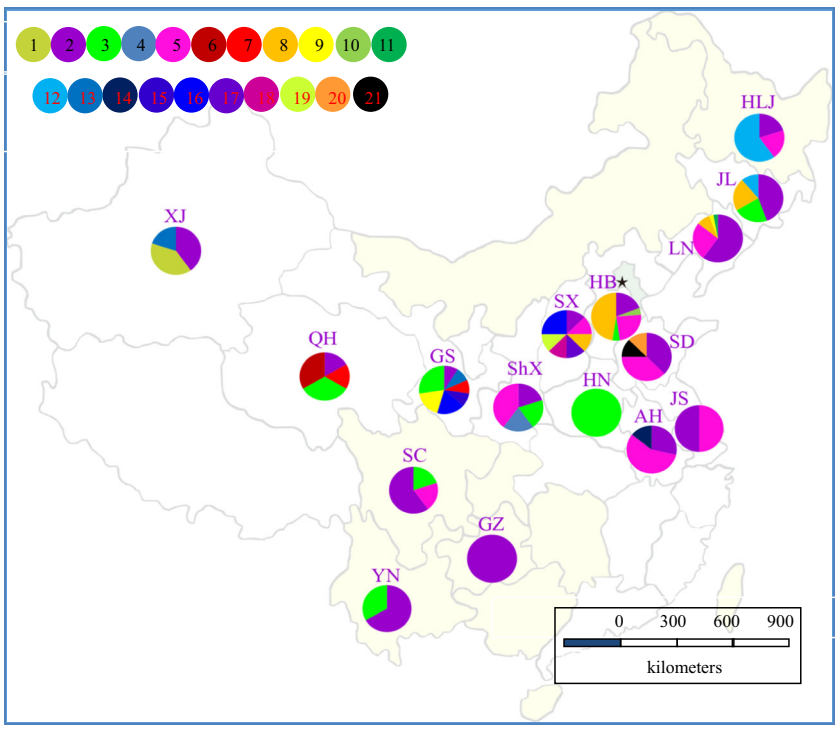

Fig. 2 The relative frequencies and geographic distributions of 21 haplotypes in 16 provinces of China. The 21 haplotypes H_1–H_21 are represented by 21 different colours and 21 numbers (*1–21*, see legend). *AH* Anhui, *GS* Gansu, *GZ* Guizhou, *HB* Hebei, *HLJ* Heilongjiang, *HN* Henan, *JL* Jilin, *JS* Jiangsu, *LN* Liaoning, *QH* Qinghai, *ShX* Shaanxi, *SD* Shandong, *SX* Shanxi, *SC* Sichuan, *XJ* Xinjiang, *YN* Yunnan

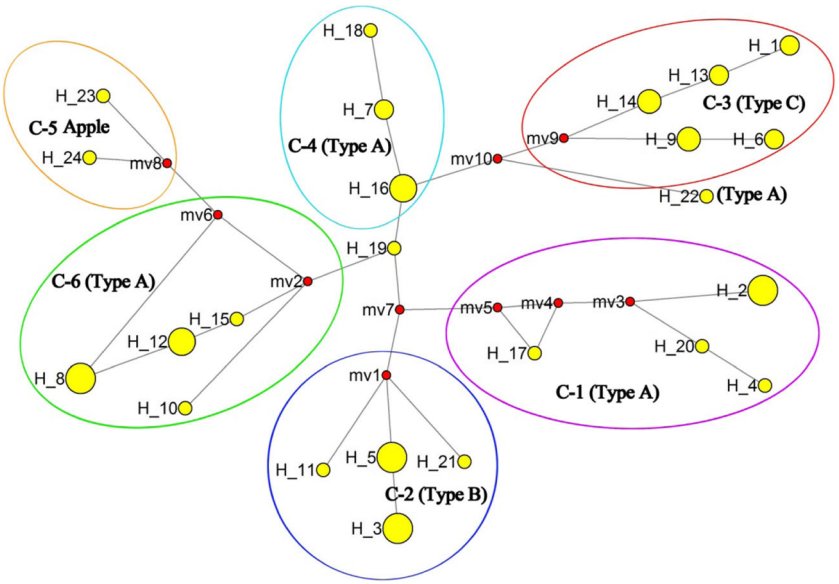

Fig. 3 Median-joining network for cpDNA haplotypes in 132 pear accessions and outgroups based on six combined chloroplast DNA regions. The haplotypes are indicated by the *yellow circles*, the size of each *circle* being proportional to the observed frequency of each haplotype. Each *circle*, each node of each haplotype and the median vectors are labelled as *C*, *H* and *mv*, respectively

haplotypes with a 141-bp deletion in the *rps*16-*trn*Q region and represented the majority of Sinkiang Pear cultivars (eight out of nine, 88.9%), H_14 was a type C haplotype with a 141-bp deletion in the *rps*16-*trn*Q region and H_22 was a type A haplotype. Both H_14 and H_22 represented all the European pears (*P. communis*) used in this study.

Discussion

Relatively abundant cpDNA diversity and haplotype diversity of pear in Northern China

The *acc*D-*psa*I and *trn*L-*trn*F intergenic spacers displayed the most polymorphic sites in the study of genetic characterization of pear varieties (Kimura et al. 2003). Consistently, in our study, the hypervariable region *trn*L-*trn*F-413 possessed the highest nucleotide diversity (P_i = 0.00233) and an average number of nucleotide differences (k = 0.91869). Moreover, the hypervariable region *acc*D-*psa*I showed the third highest values for P_i (P_i = 0.00086) and k (k = 0.61531).

It is well known that cpDNA is a maternally inherited marker that undergoes little or no recombination and exhibits high levels of genetic variations, such as insertions, deletions, translocations and inversions (Clegg and Zurawski 1992; Petit et al. 1993). Some cpDNA regions are ideal fragments for phylogenetic research, hybrid cultivar identification and genetic diversity research. CpDNA is quite conservative, and the main mutation types are point mutations and indels. The genetic diversity of pear cpDNA was quite low (Katayama and Uematsu 2003; Katayama et al. 2012). In this study, the nucleotide diversity of 132 pear accessions from Northern China was 0.00070, lower than the Pi (P_i = 0.00105) of Callery pear accessions in Zhejiang (Liu et al. 2012). However, the haplotype (gene) diversity (H_d = 0.7960) was slightly higher than that of Callery pears (H_d = 0.719). This was probably because the haplotype number (*h)* corresponding to H_d of the 132 pear trees in the study was 21, much higher than that of 10 of *P. calleryana*, indicating a relatively abundant genetic diversity of pear trees in Northern China.

Sixteen haplotypes were found in 114 Chinese pear cultivars, slightly higher than that in previous studies (Liu et al. 2012; Wuyun et al. 2013). The cultivars of *P. bretschneideri* harboured seven haplotypes (h = 7), followed by *P. ussuriensis* (h = 6) and *P. sinkiangensis* (h = 5). Both wild *P. ussuriensis* and *P. betuleafolia* accessions had five haplotypes and shared H_16, which is less than those of wild Ussurian Pear accessions in a previous study (Wuyun et al. 2013). In summary, compared to wild pears, the Chinese pear cultivars in Northern China showed a wide range of genetic diversity and haplotypes in cpDNA, consistent with the results of Wuyun et al. (2013).

Important pear cpDNA haplotypes and their relationships revealed by the median-joining network

H_19 was unique to *P. betuleafolia* 'Shanxi Duli 1' and lay in the torso of the Median-joining network (Fig. 3). Therefore, it was considered to be one of the ancient haplotypes and a divergent centre. H_16 was the joint of haplotypes in circles C-3 and C-4. In addition, it was also the shared haplotype of wild *P. ussuriensis* and *P. betuleafolia* accessions. H_2 was found in most pear germplasm resources (46 cultivars and two wild Ussurian Pear accessions), followed by haplotype H_5 (30 cultivars, two wild Ussurian Pear accessions and four sand pears in outgroups) in the cultivars 'Dangshan Su- li' and 'Yali', which

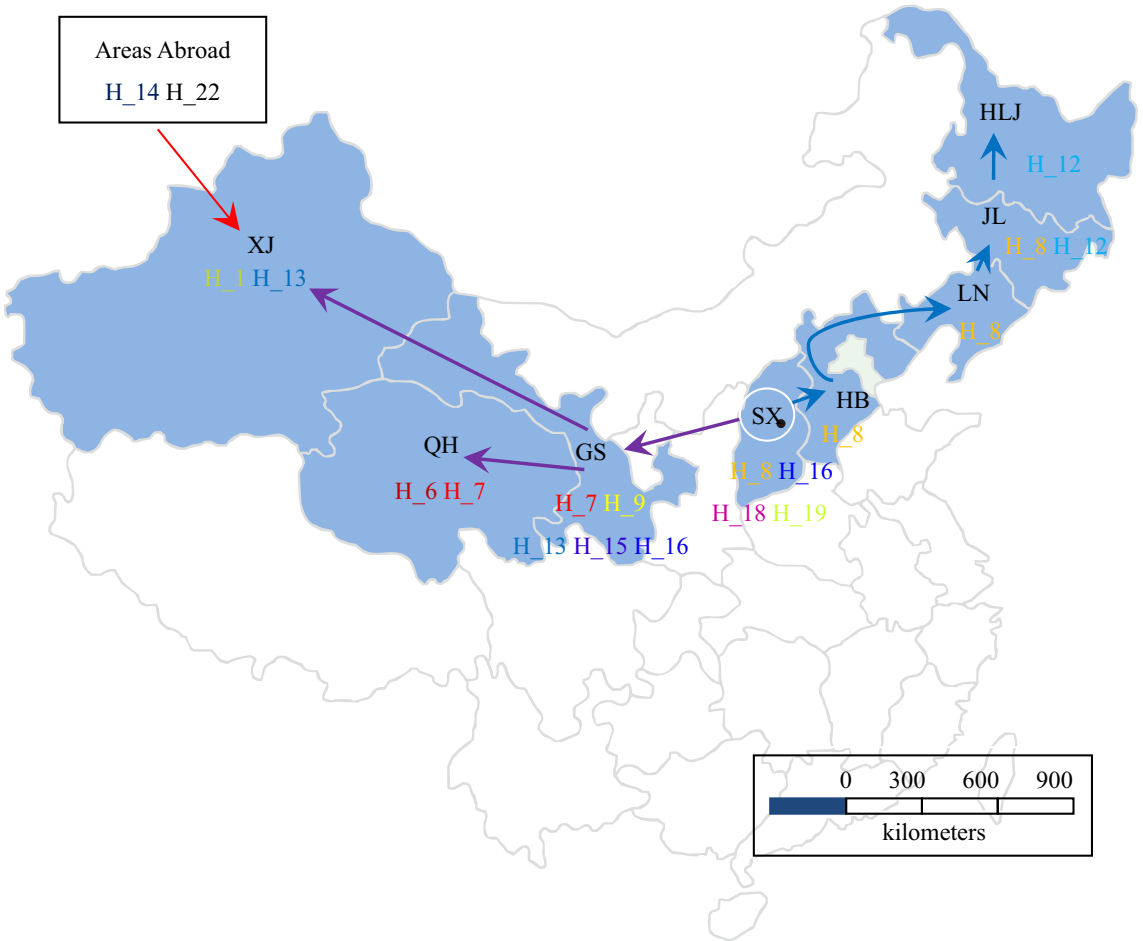

Fig. 4 The map of evolution routes of *Pyrus* in Northern China. The *arrow* shows the evolution direction. *AH* Anhui, *GS* Gansu, *GZ* Guizhou, *HB* Hebei, *HLJ* Heilongjiang, *HN* Henan, *JL* Jilin, *JS* Jiangsu, *LN* Liaoning, *QH* Qinghai, *ShX* Shaanxi, *SD* Shandong, *SX* Shanxi, *SC* Sichuan, *XJ* Xinjiang, *YN* Yunnan. The colour of each haplotype corresponds to that in Fig. 2

had the largest and second largest cultivation areas in China. Both H_2 and H_5 were ancient haplotypes of pear.

Nucleotide substitutions were also found between these haplotypes. There was one nucleotide substitution between H_4 and H_20, two between H_2 and H_20 and three between H_2 and H_4. In addition, a 2-bp indel was found between H_3 and H_5. Two singleton variable sites in cpDNA sequence were also identified among H_1, H_13 and H_14. Thus, we concluded that they had a close kinship with each other. Similarly, only one single nucleotide difference was found between H_6 and H_9. H_22 was the only haplotype that did not belong to Type C in circle C-3 and had a cpDNA sequence that was obviously different from other haplotypes determined through alignment; thus, it was spatially separated in the analyses using mv10. Therefore, it had a relatively distant relationship with the remaining haplotypes in this circle.

H_7, H_16 and H_18 in C-4 belonged to type A and had a close relationship to each other. H_7 differed from H_16 only by an A↔G transition and from H_18 by a 24 bp direct repeat region and gap (AAGAA ATAAG AATCA ACTTC TATA), in agreement with Katayama et al. (2012).

H_8 and H_15 connected to each other via H_12. H_8 represented the majority of Ussurian cultivars while H_12 represented all wild Ussurian accessions. The three haplotypes had a relatively close kinship and only varied by a 4-bp indel between H_8 and H_12 and an 18-bp indel between H_12 and H_15.

The haplotypes of occidental pears and most of the oriental pears lay in different and even opposite directions in the median-joining network, and they had obvious differences, indicating that they evolved independently and had a distant relationship (Zhang et al. 2016).

Genetic relationships of pear accessions with important haplotypes

Pear is generally considered as a complicated population without a high amount of gene flow among different species. However, our results of combined sequence analyses of six cpDNA regions showed clear genetic relationships between and within wild and cultivated accessions.

'Korla pear', a member of H_2 in this study, has been cultivated for more than 1300 years. As a famous variety in Xinjiang

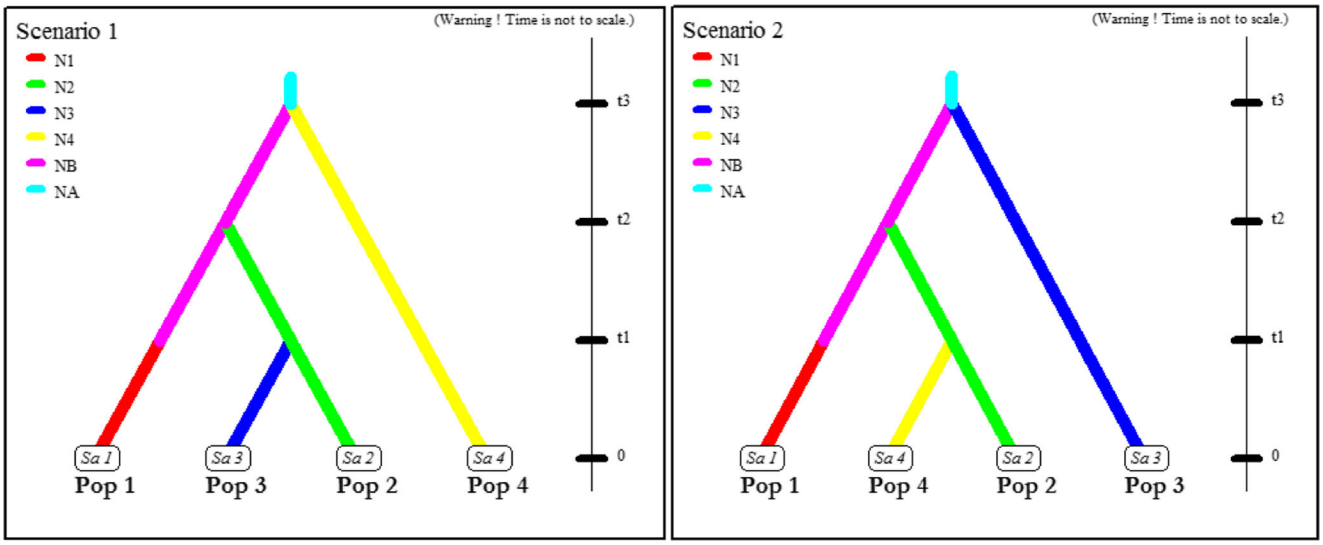

Fig. 5 Two biogeography scenarios constructed based on approximate Bayesian computation (ABC) using DIYABC. *Pop 1*, *Pop 2*, *Pop 3* and *Pop 4* mean all individuals collected from Shanxi, Gansu, Qinghai and Xinjiang, respectively. Scenario 1 means the route Shanxi → Gansu → Qinghai and scenario 2 means Shanxi → Gansu → Xinjiang. The time is set as *t3* > *t2* > *t1*

and perhaps in China, it has drawn wide attention among Chinese scholars. Some scholars believed that it is a hybrid species between occidental and white pears (Yang 1985, Zou et al. 1986, Teng et al. 2001) while others tended to attribute it to White Pear (He et al. 2011, Yang 2010). Ma et al. (2004a, b) and Shan et al. (2010) considered it as Sinkiang Pear based on their studies using ISSR and RAPD markers. We found that 'Korla pear' shared H_2 with White Pear 'Donghuang' from Xinjiang province and had haplotypes different from other Sinkiang Pear accessions, implying that White Pear participated in their evolution. In addition, considering that cpDNA is of matrilineal inheritance and reflects the matriarchal evolutionary history, we deduced that the female parent of 'Korla pear' was most likely to be *P. bretschneideri*. Apart from *P. bretschneideri* and *P. sinkiangensis* pears, the haplotype of *P. pyrifolia*, *P. ussuriensis* and *P. xerophila* pears was also H_2. Pu et al. (1985, 1986) conducted cytological studies and found that they all had triploid germplasm and displayed a similar genetic background.

Cultivars 'Yanbian Dashan' and 'Pingguoli' collected from Yanbian were identical in the combined cpDNA sequences, and both belonged to H_3. H_3 was composed of nine Chinese White Pear accessions and three Chinese Sand Pear cultivars and had a 229-bp indel fragment in cpDNA. 'Pingguoli' is one of the excellent pear varieties and has a cultivation history in China of over 90 years. Although it has been reported that 'Pingguoli' was introduced from Gyeonggido of North Korea (Wu 1984), where the major pear cultivars were anti-cold Japanese pear accessions, its origin is still debated. Based on the Yanbian Fruit Tree Survey Section (in Jilin province) in 1952, 'Pingguoli' was originally introduced to China in 1921 and named 'Lipingguo' at that time. After years of breeding and cultivation, it was renamed

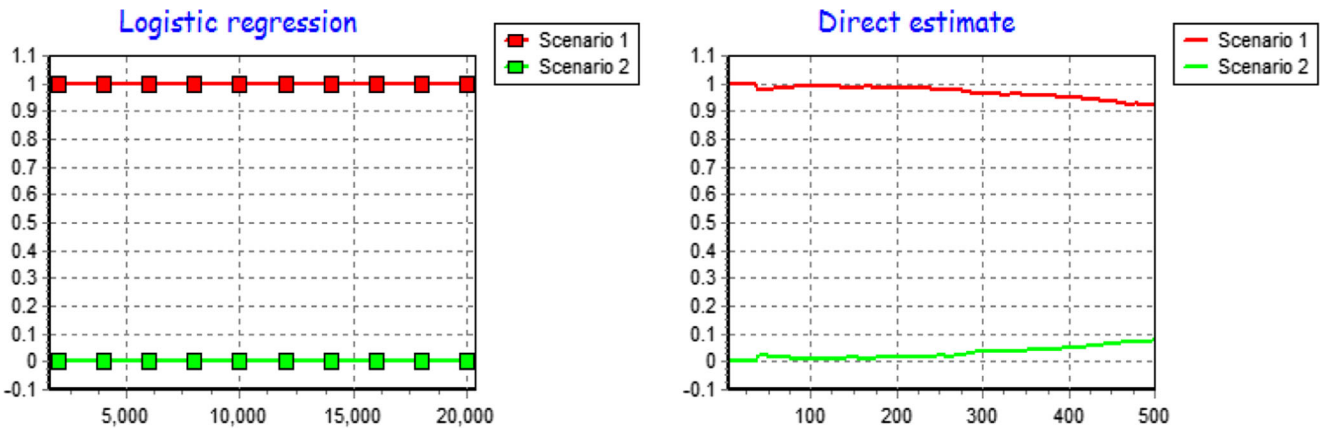

Fig. 6 Logistic regression analysis (*left*) and direct estimate (*right*) of posterior probabilities for scenario 1 and scenario 2

as 'Pingguoli' (Jing 1989; Qu et al. 2002, 2003; Yang et al. 2010). Moreover, classification of 'Pingguoli' is also controversial. Some scholars believed that 'Pingguoli' belonged to *P. bretschneideri* (Qu et al. 2001, 2002, 2003; Ma and Zhang 2009; Lu and Zhang 2009; Yang et al. 2010), while others considered it as *P. pyrifolia* (Challice and Westwood 1973) or a hybrid (Wang 1988; Qu et al. 1990; Ma et al. 2004a). Our results showed that it was clustered and shared the same combined cpDNA sequences with the cultivar 'Yanbian Dashan' from Yanbian, indicating that it had a similar genetic background with *P. pyrifolia*.

Two Japanese sand pears and 34 Chinese pears from nine provinces, including cultivars of *P. ussuriensis*, *P. bretschneideri* and *P. pyrifolia* formed H_5 and possessed type B haplotype with a 229-bp indel fragment, exhibiting a closely related relationship. Our results are consistent with previous reports showing that Japanese sand pear cultivars and Chinese sand pear cultivars shared similar genetic backgrounds and exhibited a high degree of kinship (Teng et al. 2002; Shen et al. 2006; Lu et al. 2011). In this study, Ussurian pear cultivars 'Xiaoxiangshui' and 'Yanbian Longjing' from Northeastern China had the same cpDNA sequences and belonged to H_8 together with the cultivar 'Yanbian Xiehuatian' from Yanbian of Jilin. The results are consistent with the results of Cao et al. (2012), showing that 'Xiaoxiangshui' and 'Yanbian Longjing' shared the same SSR alleles and had a relatively close relationship with 'Yanbian Xiehuatian'. Together, these results demonstrated that the above conclusion was reliable at both levels of nuclear and cpDNA genomes.

Exploration of the supposed evolution routes of *Pyrus* from the median-joining network

The haplotype of wild *P. betuleafolia* accession 'Shanxi Duli 1' from Qinyuan, Shanxi province, one of the pear divergent centres, was H_19 and located in the torso of the Median-joining network (Fig. 3). Therefore, its site of origin, Shanxi province was regarded as the starting point of the evolution routes (Fig. 4). In addition, H_19 belonged to type A, which was one of the three cpDNA types and assumed to be the most primitive cpDNA type in a previous study (Katayama et al. 2012), implying that selecting Shanxi province as the starting point of the evolution routes was feasible.

H_16 represented the haplotype of three wild *P. ussuriensis* accessions and one wild *P. betuleafolia* accession from Gansu and Shanxi. There are several possible geographical evolutionary routes such as the route Gansu → Qinghai → Shanxi based on the analysis of circle C-3 and Gansu → Xinjiang or Gansu → Qinghai based on the analysis of circle C-4. Remarkably, Gansu was considered as a vital location, especially the famous Hexi Corridor. To test which putative route was more supported, two scenarios of population divergence (Fig. 5) were constructed and evaluated based on approximate Bayesian computation (ABC) using DIYABC ver2.0 (Cornuet et al. 2014). Individuals from Shanxi, Gansu, Qinghai and Xinjiang provinces were treated as Pop 1, Pop 2, Pop 3 and Pop 4, respectively. Logistic regression computation and direct estimate were used to calculate posterior probabilities for the two scenarios (Fig. 6). Both approaches are congruent and show maximum support for the first scenario, indicating that Shanxi → Gansu → Qinghai was more likely to be the evolution route.

Haplotypes of the occidental pear were H_14 and H_22, the latter being divergent in the Median-joining network (Fig. 3). H_1 and H_6 were the haplotypes of Xinjiang pears from Xinjiang and Qinghai, respectively. Both H_9 and H_13 were the haplotypes of pears mainly from Gansu and H_13 was also a part of Xinjiang. As shown in the circle C-3 of the Median-joining network, there was a relatively close relationship between Sinkiang pear and occidental pear, consistent with another research (Liu 2006). This relationship could be further explained in the right part of Fig. 1, showing that H_14 and H_22 belonging to occidental pears merged earlier than H_1 and H_13 belonging to Xinjiang pears. Therefore, we concluded that occidental pears participated in the evolution of Xinjiang pears via geographic evolutional route of Areas Abroad → Xinjiang. Whether pear from Xinjiang also spread to foreign areas was beyond the scope of our study. Moreover, the relationship between oriental pears and occidental pears needs to be further analysed using more materials.

Another route was concluded based on the haplotype information in circle C-6. H_12 belonged to all the wild *P. ussuriensis* accessions from Jilin and Heilongjiang, whereas H_8 mainly consisted of *P. ussuriensis* accessions from Hebei, Jilin and Liaoning, including two wild *P. betuleafolia* accessions from Hebei and Shanxi. The difference between wild and cultivated Ussurian Pear accessions in Northern China was a 4-bp indel (AAAA), showing a very close relationship. Moreover, H_12 and H_8 differed from H_19 by a 10-bp indel and a 1-bp indel, respectively. These indels may be the critical force of evolution. Our results support the theory that pear trees spread from Yanshan Mountain in Hebei to Liaoning, Jilin and Heilongjiang.

In summary, to the best of our knowledge, this is the first report exploring the evolution routes of *Pyrus* based on cpDNA divergence in the background of pear phylogeny in Northern China.

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Data archiving statement The authors declare that all the work described in this manuscript followed the standard Tree Genetics and Genomes policy. The sequences will be uploaded soon, and all the sequences in this study will be found in the National Center of Biotechnology Information (NCBI) database.

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