Mol Genet Genomics (2015) 290:225–237
DOI 10.1007/s00438-014-0914-5

ORIGINAL PAPER

Prediction of retrotransposons and assessment of genetic variability based on developed retrotransposon-based insertion polymorphism (RBIP) markers in *Pyrus* L

Shuang Jiang · Yu Zong · Xiaoyan Yue · Joseph Postman · Yuanwen Teng · Danying Cai

Received: 8 May 2014 / Accepted: 2 September 2014 / Published online: 13 September 2014

Abstract Interspecific hybridization has been considered the major mode of evolution in *Pyrus* (pear), and thus, the genetic relationships within this genus have not been well documented. Retrotransposons are ubiquitous components of plant genomes and 42.4 % of the pear genome was reported to be long terminal repeat (LTR) retrotransposons, implying that retrotransposons might be significant in the evolution of *Pyrus*. In this study, 1,836 putative full-length LTR retrotransposons were isolated and 196 retrotransposon-based insertion polymorphism (RBIP) primers were developed, of which 24 pairs to the *Ppcr1* subfamily of *copia* retrotransposons were used to analyze genetic diversity among 110 *Pyrus* accessions from Eurasia. Our results showed that *Ppcr1* replicated many times in the development of cultivated Asian pears. The genetic structure analysis and the unweighted pair group method with arithmetic mean (UPGMA) dendrogram indicated that all accessions could be divided into Oriental and Occidental groups. In Oriental pears, wild pea pears clustered separately into independent groups in accordance with their morphological classifications. Cultivars of *P. ussuriensis* Maxim, *P. pyrifolia* Nakai, and *P. pyrifolia* Chinese white pear were mingled together, which inferred that hybridization events occurred during the development of the cultivated Asian pears. In Occidental pears, two clades were obtained in the UPGMA dendrogram in accordance with their geographical distribution; one contained the European species and the other included species from North Africa and West Asia. New findings in this study will be important to further understand the phylogeny of *Pyrus* and origins of cultivated pears.

Keywords Retrotransposon · *Pyrus* · RBIP marker · Insertion polymorphism · Genetic relationship

Communicated by S. Hohmann.

Electronic supplementary material The online version of this article (doi:10.1007/s00438-014-0914-5) contains supplementary material, which is available to authorized users.

S. Jiang · Y. Zong · X. Yue · Y. Teng (✉) · D. Cai
Department of Horticulture, The State Agricultural Ministry Key Laboratory of Horticultural Plant Growth, Development of Quality Improvement, Zhejiang University, Hangzhou, Zhejiang 310058, China
e-mail: ywteng@zju.edu.cn

D. Cai
e-mail: dycai@zju.edu.cn

J. Postman
National Clonal Germplasm Repository, USDA-ARS, Corvallis, OR 97333, USA

Introduction

The genus *Pyrus* L. (common name pear) is classified in the subtribe Pyrinae of the tribe Pyreae (Potter et al. 2007). These names have been corrected to Malinae and Maleae, respectively, based on recent changes to the International Code of Nomenclature for Algae, Fungi, and Plants (McNeill 2012), and about 20 primary species are generally accepted by most taxonomists. According to their original distribution areas, *Pyrus* is divided geographically into two groups: the Occidental pear and the Oriental pear (Bailey 1917). Occidental pears are further divided into three groups: West Asian species, North African species, and European species (Challice and Westwood 1973). *Pyrus communis* L. is the major cultivated species of Occidental pear, which has been produced widely throughout Europe, North and South America, and Africa.

Cultivated pears native to Asia mainly belong to four species or types (Teng and Tanabe 2004): *P. pyrifolia* Chinese white pear group (sometimes mistakenly assigned as *P. bretschneideri* Rehd.), *P. ussuriensis* Maxim., *P. pyrifolia* Nakai, and *P. sinkiangensis* Yu, which is believed to be a hybrid of Chinese white pear or Chinese sand pear and Occidental pear (Teng et al. 2001). During the last decades, molecular markers, including random amplified polymorphic DNAs (RAPDs) (Monte-Corvo et al. 2000; Teng et al. 2001, 2002), amplified fragment length polymorphisms (AFLPs) (Bao et al. 2008; Monte-Corvo et al. 2000), and simple sequence repeats (SSRs) (Bao et al. 2007; Bassil and Postman 2010; Yamamoto et al. 2002; Yao et al. 2010), have been applied in *Pyrus* species to evaluate their genetic diversity and genetic relationships. However, the genetic relationships among some *Pyrus* species are still unclear owing to the extensive interspecific hybridization in *Pyrus* (Zheng et al. 2008, 2011, 2014). Recently, 42.4 % of the pear genome was reported to be long terminal repeat (LTR) retrotransposons (Wu et al. 2013), which implied that retrotransposons might be closely related to the evolution of *Pyrus*. Therefore, the development of new retrotransposon-based markers could be indispensable in further understanding the genetic relationship and origin of *Pyrus*.

Retrotransposons are a widespread class of transposable elements that exist in all plant species investigated to date (Sabot and Schulman 2006; SanMiguel et al. 1996; Wicker et al. 2007). They replicatively transpose by way of an RNA intermediate, and thus their copy numbers increase and occupy large fractions of the genomes, especially in higher plants. For example, 7.5 and 37.6 % of the *Arabidopsis* and apple genomes are estimated to be retrotransposons, respectively (Velasco et al. 2010). LTR retrotransposons is one of the most important retrotransposon families (Havecker et al. 2004; Peterson et al. 2002; SanMiguel et al. 1998; Vicient et al. 1999). LTRs are easy to find because of their presence as flanking sequences at the 5′ and 3′ ends of coding regions in the genome (Bergman and Quesneville 2007). They are suitable for developing new molecular markers because of their ubiquitous distribution, abundant copy number, and insertion polymorphisms (Flavell et al. 1992). Recently, retrotransposon-based markers, such as retrotransposon-based insertion polymorphisms (RBIPs) (Kalendar et al. 2011), inter-retrotransposon amplified polymorphisms (IRAPs) (Kalendar and Schulman 2006), retrotransposon-microsatellite amplified polymorphisms (REMAPs) (Kalendar and Schulman 2006), and sequence-specific amplification polymorphisms (SSAPs) (Waugh et al. 1997) have been developed in some species and widely used in studies of genetic diversity, phylogeny, genetic mapping, and cultivar identification.

RBIP identification involves the simple PCR-based detection of retrotransposon insertions using primers that flank the insertion site and primers from the insertion itself (Kalendar et al. 2011). Researchers have tried to develop RBIP markers in Rosaceae, but these efforts were constrained by the lack of known LTR sequences. Jing et al. (2005) carried out SSAP PCRs to obtain 52 RBIP markers, which are costly and time consuming. Kim et al. (2011, 2012) first isolated retrotransposons from a BAC library in *Pyrus* and developed 22 RBIP makers using BLASTN sequence similarity searches based on the LTR sequence of the *copia*-like retrotransposon *Ppcrt*4. Using these markers, 61 of 64 Japanese pear cultivars could be distinguished. However, a BAC library is too limited to identify the retrotransposons in pear, and the sequence homology analysis by BLASTN could not identify all the retrotransposons, especially retrotransposons that were specific to pear. Recently, the whole genome of *Pyrus* (*Pyrus bretschneideri* Rehd. cv. 'Dangshansuli') has been sequenced (Wu et al. 2013), which has opened the way for a faster and more economical approach to predicting retrotransposons and developing RBIP markers.

In the current study, we aimed to develop new RBIP markers across the whole pear genome using LTR harvest (Ellinghaus et al. 2008). We also validated 24 primers in 110 pear accessions covering nearly all species of the genus *Pyrus* to evaluate the genetic relationships among *Pyrus* species. The results will give new insights into the genetic diversity within the *Pyrus* species.

Materials and methods

Plant materials and DNA extraction

A total of 110 *Pyrus* accessions from Eurasia were used (Table 1). Accessions of Oriental pears were from China Pear Germplasm Repository, Xingcheng, Liaoning Province, China (CPGR), Gansu Pomology Institute, Gansu Academy of Agricultural Sciences, Lanzhou, Gansu Province, China (GPI), Tottori University, Tottori, Japan (TU), Wuhan Sand Pear Germplasm Repository, Wuhan, Hubei Province, China (WSGR), Zhengzhou Fruit Institute, Chinese Academy of Agricultural Sciences, Zhengzhou, Henan Province, China (ZZFI), and field collections. All accessions of Occidental pears were collected from the National Clonal Germplasm Repository, Corvallis, OR, USA (NCGR).

Total genomic DNA was extracted from the leaf tissues of the plants following the modified CTAB protocol described by Doyle and Doyle (1987). The DNA concentrations were diluted to 10–30 ng μL^{-1} after the quality and quantity were determined on 1 % (wv^{-1}) agarose gels using standard DNA markers (Takara, Dalian, China).

Table 1 *Pyrus* species and cultivars used in this study

Pyrus sp. or cultivar[a]	Origin	Leaf source[b]
Cultivars of Chinese white pear (*P. pyrifolia* CWPG)		
Cili	Shandong, China	CPGR
Dadongguo	Gansu, China	GPI
Dangshansuli	Anhui, China	CPGR
Dawowo	Shanxi, China	Field collection
Eli	Henan, China	CPGR
Fenhongxiao	Hebei, China	CPGR
Fengxianjitui	Shanxi, China	CPGR
Haitangsu	Jiangsu, China	CPGR
Huangjitui	Jiangsu, China	CPGR
Jingchuan	Gansu, China	CPGR
Qingpicao	Anhui, China	CPGR
Xiangchun	Shanxi, China	CPGR
Xuehua	Hebei, China	CPGR
Yali	Hebei, China	TU
Yinbai	Hebei, China	CPGR
Yuanlingli	Shanxi, China	Field collection
Chinese sand pear (*P. pyrifolia*)		
Baozhuli	Yunnan, China	WSGR
Baiwali	Guizhou, China	Field collection
Dahuangcha	Zhejiang, China	WSGR
Dalinaitou	Yunnan, China	WSGR
Damali	Sichuan, China	WSGR
Heipili	Guangxi, China	Field collection
Hongpisu	Sichuan, China	WSGR
Hongshaobang	Sichuan, China	WSGR
Huahong	Zhejiang, China	WSGR
Huangpitangli	Guangxi, China	Field collection
Huobali	Yunnan, China	WSGR
Jiuzhong	Zhejiang, China	WSGR
Muguali	Guizhou, China	Field collection
Mandingxueli	Fujian, China	WSGR
Puguali	Zhejiang, China	WSGR
Rentouli	Zhejiang, China	WSGR
Shengxianshali	Zhejiang, China	WSGR
Shuinanli	Guangxi, China	Field collection
Wuli	Guizhou, China	Field collection
Yandangxueli	Zhejiang, China	WSGR
Yiwulizi	Zhejiang, China	WSGR
Japanese pear (*P. pyrifolia*)		
Babauchi	Kyushu, Japan	TU
Chojuro	Kanagawa, Japan	TU
Hakataao	Fukuoka, Japan	TU
Hakuteiryu	Niigata, Japan	TU
Imamuraaki	Kochi, Japan	TU
Ichiharawase	Kochi, Japan	TU
Kawauchikoboku	Hiroshima, Japan	TU
Nijisseiki	Chiba, Japan	TU
Tsukutonashi	Kyushu, Japan	TU
Umajirou	Kochi, Japan	TU
Cultivars of *P. ussuriensis*		
Balixiang	Liaoning, China	CPGR
Daxiangshui	Liaoning, China	CPGR
Hongbalixiang	Liaoning, China	CPGR
Huagai	Liaoning, China	CPGR
Jianbali	Liaoning, China	CPGR
Mangyuanxiang	Liaoning, China	CPGR
Nanguoli	Liaoning, China	CPGR
Qingmian	Hebei, China	CPGR
Ruanerli	Gansu, China	GPI
Tianqiuzi	Liaoning, China	CPGR
Xiaoxiangshui	Liaoning, China	CPGR
Xiehuatian	Liaoning, China	CPGR
Yaguang	Hebei, China	CPGR
Cultivars of *P. sinkiangenesis*		
Korlaxiangli	Xinjiang, China	Field collection
Wild pears originating from East Asia		
P. ussuriensis	Northeast China	CPGR
P. phaeocarpa	North China	CPGR
P. hondoensis	Middle Japan	TU
P. hopeiensis	Hebei, China	Field collection
P. betulaefolia 1	Shandong, China	Field collection
P. betulaefolia 2	Henan, China	Field collection
P. betulaefolia 3	Hebei, China	Field collection
P. betulaefolia 4	Shaanxi, China	Field collection
P. dimorphophylla 4	Mie, Japan	TU
P. dimorphophylla 5	Mie, Japan	TU
P. calleryana 1	South China	TU
P. calleryana 2	Liaoning, China	CPGR
P. pashia 1	Yunnan, China	Field collection
P. pashia 2	Yunnan, China	Field collection
P. pashia 3	Yunnan, China	Field collection
P. pashia 4	Yunnan, China	Field collection
P. pashia 5	Yunnan, China	Field collection
P. pashia 6	Yunnan, China	Field collection
P. pashia 7	Yunnan, China	Field collection
P. xerophila 1	Gansu, China	GPI
P. xerophila 2	Gansu, China	GPI
P. xerophila 3	Gansu, China	GPI
Cultivars *of P. communis*		
Cascade	USA	ZZFI
Comice	France	ZZFI
Species originating from Europe		
P. caucasica 684	Ukraine	NCGR
P. caucasica 687	Russia	NCGR
P. caucasica 694	Russia	NCGR
P. caucasica 2816	Armenia	NCGR
P. pyraster 989	Yugoslavia	NCGR

Table 1 continued

Pyrus sp. or cultivar[a]	Origin	Leaf source[b]
P. pyraster 1288	Iran	NCGR
P. pyraster 1671	Romania	NCGR
P. cordata 1588	Turkey	NCGR
P. nivalis 256	Netherland	NCGR
Species originating from West Asian		
P. elaeagrifolia 768	Turkey	NCGR
P. elaeagrifolia 1490	Turkey	NCGR
P. spinosa 1598	Italy	NCGR
P. regelii 890	Russia	NCGR
P. regelii 2513	Kazakhstan	NCGR
P. salicifolia 2720	Russia	NCGR
P. salicifolia 2797	Armenia	NCGR
P. syriaca 908	Israel	NCGR
Species originating from North African		
P. cossonii 753	Morocco	NCGR
P. cossonii 828	uncertain	NCGR
P. gharbiana 789	Morocco	NCGR
P. gharbiana 790	Morocco	NCGR
P. gharbiana 794	Morocco	NCGR
P. mamorensis 834	Morocco	NCGR
P. mamorensis 835	Morocco	NCGR
P. mamorensis 837	Morocco	NCGR

[a] Classification of species and cultivars from Yu (1979), and Challice and Westwood (1973)

[b] CPGR: China Pear Germplasm Repository, Xingcheng, Liaoning Province, China; GPI: Gansu Pomology Institute, Gansu Academy of Agricultural Sciences, Lanzhou, Gansu Province, China; NCGR: National Clonal Germplasm Repository, USA; TU: Tottori University, Japan; WSGR: Wuhan Sand Pear Germplasm Repository, Wuhan, Hubei Province, China; and ZZFI: Zhengzhou Fruit Institute, Chinese Academy of Agricultural Sciences, Zhengzhou, Henan Province, China

Identification of LTR retrotransposons

The whole genome of *P. bretschneideri* Rehd. cv. 'Dangshansuli' (AJSU00000000) was used to predict retrotransposons. LTR harvest was run on the whole genome data using default settings to isolate LTR retrotransposons based on certain features, including two nearly identical LTR sequences flanked by target site duplications (TSDs) of 4–6 bp and some conserved sequence motifs, such as the poly purine tract (PPT) and the primer binding site (PBS) (Fig. 1a). The genomic sequences between any two putative LTRs were subsequently analyzed using BLASTN searches (the *E* value threshold was 10^{-5}) against the GenBank non-redundant databases and repeat masking (Smit et al. 1996-2004) against Repbase (Kohany et al. 2006) to assign the putative LTR retrotransposons to the *copia* or *gypsy* family. The putative LTR retrotransposons in these two families were clustered (sequence length to be covered 70 %; percentage identity threshold 80 %) using Blastclust (Dondoshansky and Wolf 2002) to further classify the LTRs into the subfamilies. These LTR regions of the subfamilies were used to develop the RBIP primers.

RBIP primer design

The LTR sequences in each subfamily were first aligned using ClustalW software (Larkin et al. 2007) to obtain conserved sequences and were then mapped to the pear genome using BLAST. Primer sets were designed using Primer3 software (Koressaar and Remm 2007) to amplify the junction regions between the 3′ LTR retrotransposon sequences and the flanking pear genome sequences (Fig. 1a, b). One primer in each set was designed within the 3′ LTR of the retrotransposon and labeled with a fluorescent chemical (FAM, HEX, PET, or NED); the other primer was designed within the pear genomic region.

PCR amplification and analysis

Twenty-four RBIP primer pairs from the *Ppcr*1 subfamily of *copia* retrotransposons were used for DNA profiling. PCR amplifications were carried out in a total volume of 15 μL [10 ng DNA template, 0.4 μM of each primer, 200 μM dNTPs, 2 mM $MgCl_2$, and 0.5 U Taq DNA polymerase (Takara, Dalian, China)]. Amplifications were performed in a Mastercycler gradient PCR thermocycler (Eppendorf, Hamburg, Germany) programmed for an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 40 s, and then 72 °C for 6 min. Amplicons were pooled together with an internal size standard (GeneScan™ 500 LIZ; Applied Biosystems, Foster City, CA, USA) according to distinct dyes and expected fragment sizes, and subsequently separated and sequenced using an ABI 3700XL Genetic Analyzer (Applied Biosystems). Accessions were scored at each locus for the presence (1) or absence (0) of bands of the expected size.

Data analysis

The identification of a genetically homogeneous group in a data set is always challenging and is affected by several factors including the number of loci used, the magnitude and scale of gene flow, the variation at each locus, and the number of samples (Evanno et al. 2005). Therefore, we used two complementary methods to estimate the numbers of clusters at both non-hierarchical and hierarchical levels.

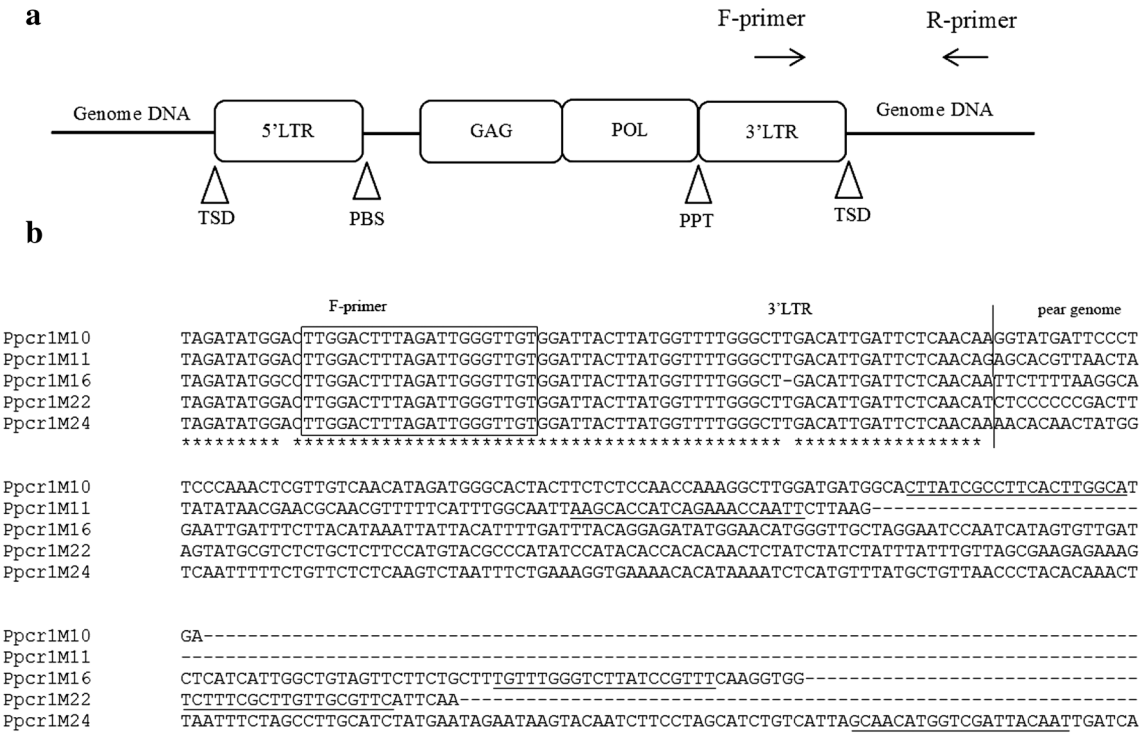

Fig. 1 Identification of LTR retrotransposons and RBIP primer design. **a** Characteristics of retrotransposons. *TSD* target site duplication, *LTR* long terminal repeat, *PBS* primer binding site, *GAG* gag protein domains, *POL* pol protein domains, *PPT* polypurine tract, *F-primer* forward primer, and *R-primer* reverse primer. **b** Alignment of five sequences between the 3′ LTR of *Ppcr*1 and the flanking sequences in the pear genome. F-primers are *boxed*; R-primers are *underlined*

For non-hierarchical genotypic clustering, the number of homogeneous genepools (*K*) was modeled using the genotypes obtained from all 110 individuals in the software STRUCTURE version 2.3.3 (Pritchard et al. 2000). This revealed the genetic structure by assigning individuals or predefined groups to *K* clusters. Ten runs of STRUCTURE were performed by setting the number of clusters (*K*) from 1 to 10. Each run consisted of a burn-in period of 200,000 iterations followed by 200,000 Monte Carlo Markov Chain iterations, assuming an admixture model. The results were uploaded to the STRUCTURE HARVESTER web site (Earl and VonHoldt 2012) to estimate the most appropriate *K* value. The replicate cluster analysis of the same data resulted in several distinct outcomes for estimated assignment coefficients, even though the same starting condition was used. Therefore, we employed CLUMPP software (Jakobsson and Rosenberg 2007) to average the 10 independent simulations and illustrated the result graphically using DISTRUCT (Rosenberg 2004).

All data were used to calculate Dice's similarity coefficients (Nei and Li 1979). An unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on the similarity matrix was constructed using the NTSYS-pc program (Version 2.10e) (Rohlf 1998).

Results

Identification and classification of LTR retrotransposons

Putative LTR retrotransposons were identified from the pear genome using LTR harvest. First, we identified all the sequences that were flanked by two highly similar sequences (>90 %) between 100 and 1,000 bp in length. Using these criteria, 1,836 full-length LTR retrotransposons were mined, making up 2.3 % of the pear genome (size 512 Mb). The identified sequences were then masked against the Repbase database using RepeatMasker to classify the retrotransposons into different families. A total of 689 retrotransposons (37.5 %) were assigned to the Ty1-*copia* family, 353 retrotransposons (19.2 %) were assigned to the Ty3-*gypsy* family, and 794 retrotransposons were unclassified and, therefore, excluded from the subsequent analysis (Table 2). The *copia* retrotransposons (689) were further clustered into 195 subfamilies of which 53 % contained a single sequence, 15 % contained two sequences, and 32 % contained three or more sequences. The *gypsy* retrotransposons (353) were further clustered into 193 subfamilies of which 84 % contained a single sequence, 8 % contained two sequences, and 8 % contained three or more sequences.

Table 2 Number of putative full-length LTR retrotransposons in the pear genome

Classification	Copies (no.)	Total length (Mb)	TE coverage (%)	Total genome coverage (%)
LTR/*copia*	689	3.6	37.5	0.7
LTR/*gypsy*	353	2.9	19.2	0.6
Unclassified	794	5.2	43.3	1.0
Total	1,836	11.7	100	2.3

TE transposable elements

Development and assessment of RBIP markers

To obtain more insertion sites, the conserved LTRs of the top five *copia* and *gypsy* subfamilies (KF806690–KF806699) were mapped against the *Pyrus* genome using BLAST to locate them in the genome and to obtain their flanking regions, which were used to develop markers (Table 3). A total of 196 RBIP primer sets were designed to amplify the specific junction regions between the LTR sequences and their flanking sequences (Fig. 1). The sizes of the PCR products were estimated to vary from 113 to 534 bp. Twenty-four RBIP primer pairs from *Ppcr*1 were used for DNA profiling (Table 4), and only the forward primer in the 3′ LTR region was designed with fluorescent chemicals to save costs. Additional RBIP primers developed from other retrotransposon subfamilies are listed in Supplementary data 1.

All of the 24 loci were polymorphic in our preliminary data analysis and were thus employed for the analysis of the main data set (Supplementary data 2). The data matrix contained 2,640 data points. All the primers showed single clear amplified bands of the expected sizes in 'Dangshansuli'. Seven primers (*Ppcr*1M3, *Ppcr*1M8, *Ppcr*1M10, *Ppcr*1M14, *Ppcr*1M15, *Ppcr*1M16, and *Ppcr*1M23) amplified fragments in more than 50 % of the pear accessions. *Ppcr*1M3 successfully generated amplified fragments in all pear accessions except two Japanese pear cultivars ('Chojuro' and 'Hakataao'). *Ppcr*1M8 amplified fragments in *P. pyrifolia* Chinese white pear group, *P. pyrifolia* and *P. ussuriensis*, but not in *P. betulaefolia* and *P. communis*. Primer *Ppcr*1M10 amplified fragments within all Oriental pear accessions except 'Daxiangshui', 'Xiaoxiangshui', 'Korlaxiangli', and '*P. xerophila*'. Three primers (*Ppcr*1M14, *Ppcr*1M15, and *Ppcr*1M16) produced amplicons mainly in Oriental pears and rarely in Occidental pears. *Ppcr*1M23 amplified fragments in most Oriental pear cultivars, but not in Occidental pears or Oriental wild pear. *Ppcr*1M20 amplified fragments in accessions originating from Europe, and *Ppcr*1M4 produced amplicons in accessions originating from West Asia and North Africa. Six primers (*Ppcr*1M1, *Ppcr*1M2, *Ppcr*1M7, *Ppcr*1M12, *Ppcr*1M18, and *Ppcr*1M22) failed to produce fragments in the Occidental pear species. Out of all the primers, *Ppcr*1M1 and *Ppcr*1M5 amplified fragments only in a few accessions.

Genetic relationships among *Pyrus* species and cultivars

Bayesian modeling of the number of homogeneous genepools (K) in STRUCTURE was used to infer the genetic structure of the *Pyrus* accessions. An evaluation of the optimum number of K following the procedure described by Evanno et al. (2005) indicated two clear optimal values for ΔK, at $K = 2$ and 4 (Fig. 2), suggesting that a model with two genepools captured a major split in the data and that substantial additional resolution was provided under a model with $K = 4$. Barplots of the proportional allocations in STRUCTURE to each genepool for $K = 2$ and 4 are shown in Fig. 3. The plots showed that these two models were related to each other hierarchically, such that the green cluster in the two-genepool model was subdivided into two (blue and red) genepools in the four-genepool model.

The primary split in the data ($K = 2$) divided the accessions into two clusters. One cluster (yellow in Fig. 3) included Oriental pear cultivars belonging to *P. pyrifolia* Chinese white pear group, *P. pyrifolia and P. ussuriensis*. The other cluster (green) was comprised of major samples from *P. betulaefolia*, *P. xerophila*, and Occidental pears. The accessions that demonstrated a high level of admixture belonged to *P. pashia and P. calleryana.* The model with four genepools was also supported by the STRUCTURE results. Under this model, the green cluster in the model of $K = 2$ was further divided into two genepools (Fig. 3). One (red) contained the wild Asian pears including *P. pashia*, *P. betulaefolia*, *P. dimorphophylla*, and *P. calleryana*. The other (blue) was composed of the Occidental pears and an Oriental species, *P. xerophila*. The remaining two genepools, which contained cultivars of *P. pyrifolia* Chinese white pear group, *P. ussuriensis* and *P. pyrifolia*, overlapped substantially with one another, and the hierarchical levels in these two clusters could hardly be recognized. In the *P. pyrifolia* Chinese white pear group, all accessions appeared to be from the two major genepools. Several accessions in *P. ussuriensis* and *P. pyrifolia* showed admixed origins, such as 'Baozhuli' with three major genepools and 'Yaguang' with four genepools. Some Occidental accessions, such as 'Cascade', 'Comice', '*P. spinosa* 1598' and '*P. regelii* 2513' should be noted because they displayed admixtures of the genepool.

An UPGMA dendrogram was generated from the genetic similarity values to elucidate the genetic relationships

Table 3 Top five subfamilies in the *copia* and *gypsy* families in *Pyrus*

Family	Subfamily	Number of retrotransposons
copia	*Ppcr*1	37
	*Ppcr*2	26
	*Ppcr*3	18
	*Ppcr*4	17
	*Ppcr*5	10
gypsy	*Ppgr*1	33
	*Ppgr*2	32
	*Ppgr*3	17
	*Ppgr*4	16
	*Ppgr*5	12

among *Pyrus* species and cultivars (Fig. 4). Oriental pear accessions and Occidental pear accessions had the lowest genetic similarity values, while most of the cultivars from Asia shared relatively closer affinities. The dendrogram could be divided into two major groups. The majority of Group 1 was composed of Oriental pear accessions, including cultivars and wild accessions, and two Occidental cultivars, 'Cascade' and 'Comice', while Group 2 included the Occidental species and an Oriental species, *P. xerophila*. This was similar to the STRUCTURE results at $K = 2$ that classified all pear accessions into two groups, but the Oriental wild species were clustered with the Occidental pears (Fig. 3). However, in the model of $K = 4$, the majority of Oriental wild pears was separated from the Oriental pear cultivars and Occidental species.

Within Group 1, four subgroups (I–IV) could be identified (Fig. 4). The dendrogram indicated the *P. pyrifolia* Chinese white pear group, *P. ussuriensis* and *P. pyrifolia*, were generally mingled and concentrated in subgroup I. Two *P. communis* cultivars, 'Cascade' from the USA and 'Comice' from France, clustered with 'Korlaxiangli' in subgroup II (Fig. 3). Subgroup III consisted of *P. calleryana*, *P. pashia*, and *P. dimorphophylla*. In subgroup IV, four samples of *P. betulaefolia* were clustered together. In Group 2, three subgroups (V–VII) were identified. Accessions of *P. xerophila* native to China clustered into subgroup V. Subgroup VI included pear species from Europe, and subgroup VII consisted of species from West Asia and North Africa, except *P. cordata*, which originated from Europe.

Discussion

Isolation and characterization of LTR retrotransposons

Genome data enable the prediction of retrotransposons that were previously unreachable. Recently, LTR retrotransposons have been predicted in many plants using genome data. For example, 2,226 LTR retrotransposons were found in the rice genome (Baucom et al. 2009) and 1,479 LTR retrotransposons were identified in the *Populus* genome (Cossu et al. 2012). Recently, with the completion of the Chinese white pear genome, 42.4 % of the genome was reported to be LTR retrotransposons (Wu et al. 2013), while 2.3 % of the pear genome was identified to be full-length LTR retrotransposons in the present study. The obvious difference in the number of LTRs (42.4 vs. 2.3 %) might be attributed to the different approaches and parameters that were used in the two studies. Additionally, only putative full-length LTR retrotransposons with two very similar LTR sequences were isolated in this study. Our results showed that full-length *copia* retrotransposons were more common than *gypsy* retrotransposons in the pear genome (Table 2). The numbers of full-length LTR retrotransposons in the different subfamilies were generally low, and *gypsy* subfamilies had more single sequences than the *copia* subfamilies. Only 10 subfamilies contained more than 10 LTR retrotransposons (Table 3). These findings are consistent with the results reported in other plants with different genome sizes (Schnable et al. 2009; Cavallini et al. 2010; Cossu et al. 2012).

Characteristics of RBIP markers and diversity of LTR retrotransposons in *Pyrus*

New bioinformatics software offers exciting perspectives for the development of new markers based on whole genome sequences. In the pear genome, the most abundant retrotransposon families were *gypsy* and *copia*, accounting for 25.5 and 16.9 % of the genome, respectively. However, simple sequence repeats contribute only 0.22 % of the genome (Wu et al. 2013), which indicated that RBIP markers were more ubiquitous than SSR markers in pear. Although a large number of SSR markers have been developed from pear genomic DNA (Fan et al. 2013), almost all the SSRs have di-nucleotide repeat motifs, and "stutter bands" are frequent (Diwan and Cregan 1997). In addition, repeat instability and an increased mutation rate in repeat lengths might be a problem for SSR markers (Wierdl et al. 1997; Yamada et al. 2002), and these do not occur in RBIP markers. RBIP markers also allow DNA profiling and the evaluation of genetic diversity, which was similar with SSR markers. However, RBIP markers amplify a single locus in samples, which is different from SSR markers that potentially amplify two or possibly more homologous loci. Compared with other retrotransposon-based markers (IRAP, REMAP, and SSAP), which display polymorphisms in band sizes owing to retrotransposon insertion, RBIP markers can detect the presence or absence of the retrotransposons in a locus produced by the integration of an element (Kalendar et al. 2011).

Table 4 RBIP primers designed from the *Ppcr*1 subfamily of *copia* retrotransposons in *Pyrus*

Primer name	F-primer	R-primer	Target size (bp)	Position in the *Pyrus* scaffolds
*Ppcr*1M1	TTGGACTTTAGATTGGGTTGT	AACGCGGATAGACAGGAAGC	359	AJSU01000206 (31730–32749 bp)
*Ppcr*1M2		CGGTGCAGGCCAATCCTTAT	256	AJSU01006861 (60980–61879 bp)
*Ppcr*1M3		TGGCTGGTACAGTTGATGAGT	367	AJSU01013458 (11112–11951 bp)
*Ppcr*1M4		AGCAAGGACCCACCCTACCG	229	AJSU01013470 (28215–29114 bp)
*Ppcr*1M5		ACTAAGGCCAAATCGGATAA	258	AJSU01013453 (1452–613 bp)
*Ppcr*1M6		TGCTATCTCAGTTGCCTTTG	318	AJSU01002497 (14373–13474 bp)
*Ppcr*1M7		AATTGGAGTGGCTTCGCAAG	298	AJSU01012954 (17673–16774 bp)
*Ppcr*1M8		CCTTACTATTGCTTGGCTTA	295	AJSU01010089 (8529–9428 bp)
*Ppcr*1M9		GCCCAAATGGGTCAAACTCG	318	AJSU01002517 (9957–9058 bp)
*Ppcr*1M10		TGCCAAGTGAAGGCGATAAG	160	AJSU01012527 (8251–9210 bp)
*Ppcr*1M11		ATTGGTTTCTGATGGTGCTT	130	AJSU01000035 (32243–33202 bp)
*Ppcr*1M12		AGGATTCCCTCGCCTTGCTC	214	AJSU01005714 (51206–52105 bp)
*Ppcr*1M13		GAGGAGCAACCGAACCAAGA	260	AJSU01007346 (1526–627 bp)
*Ppcr*1M14		GTTGTCCTTGCTTGCCTTTA	300	AJSU01007348 (8995–8096 bp)
*Ppcr*1M15		ATTCTTCCAAGGTTCGCATT	227	AJSU01008414 (38446–37547 bp)
*Ppcr*1M16		AAACGGATAAGACCCAAACA	208	AJSU01000545 (11800–10901 bp)
*Ppcr*1M17		AAAGTTAAACCTGATGTGGC	337	AJSU01016656 (4929–4030 bp)
*Ppcr*1M18		AAACCAGTAGAAGGGAGGGA	335	AJSU01014547 (27691–26792 bp)
*Ppcr*1M19		ATGGCAAGAAGAAGAGGACGA	386	AJSU01016878 (12377–13276 bp)
*Ppcr*1M20		CACGCTAATGGGAAGGAGAA	334	AJSU01025413 (5215–4316 bp)
*Ppcr*1M21		TCGGCATACAACAAGCACAT	333	AJSU01010498 (23510–24409 bp)
*Ppcr*1M22		GAACGCAACAAGCGAAAGAC	180	AJSU01001599 (36832–37731 bp)
*Ppcr*1M23		AAACTTTGGCTATTCCTCTT	297	AJSU01019552 (6598–5699 bp)
*Ppcr*1M24		ATTGTAATCGACCATGTTGC	241	AJSU01001726 (34220–33321 bp)

F-primer forward primer, *R-primer* reverse primer

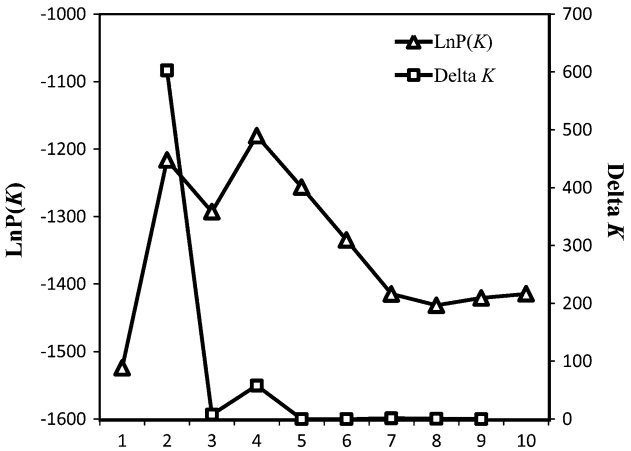

Fig. 2 Modeling of cluster number for *Pyrus* using STRUCTURE. LnP (*K*) and Delta *K* were calculated in accordance with the method of Evanno et al. (2005)

In our developed RBIP markers, each primer was related to the insertion of *Ppcr1* retrotransposons in a particular locus. Our results showed that *Ppcr*1 LTR retrotransposons existed in all pear species, even if the species were believed to be ancestral to *Pyrus*, such as *P. betulaefolia* and *P. calleryana* (Zheng et al. 2011), which suggested that *Ppcr1* retrotransposons widely existed in the pear species for a long time. In the wild Asian pears and Occidental pears, several primers did not amplify fragments, suggesting that *Ppcr1* retrotransposons were not found in these loci. However, the insertion of *Ppcr1* retrotransposons was extensively detected in many Asian pear cultivars. These results implied that *Ppcr1* retrotransposons replicated many times in the development of cultivated Asian pears, which might be one reason why 42.4 % of the genome was reported to be LTR retrotransposons in 'Dangshansuli'.

Genetic relationships among *Pyrus* species

This study was the first report to assess the genetic relationships of nearly all species of *Pyrus* based on DNA markers. The results of STRUCTURE and the dendrogram were in good agreement with the previous results of Oliveira et al. (1999), Monte-Corvo et al. (2000), Teng et al. (2001, 2002), and Bao et al. (2007, 2008), who, using a limited

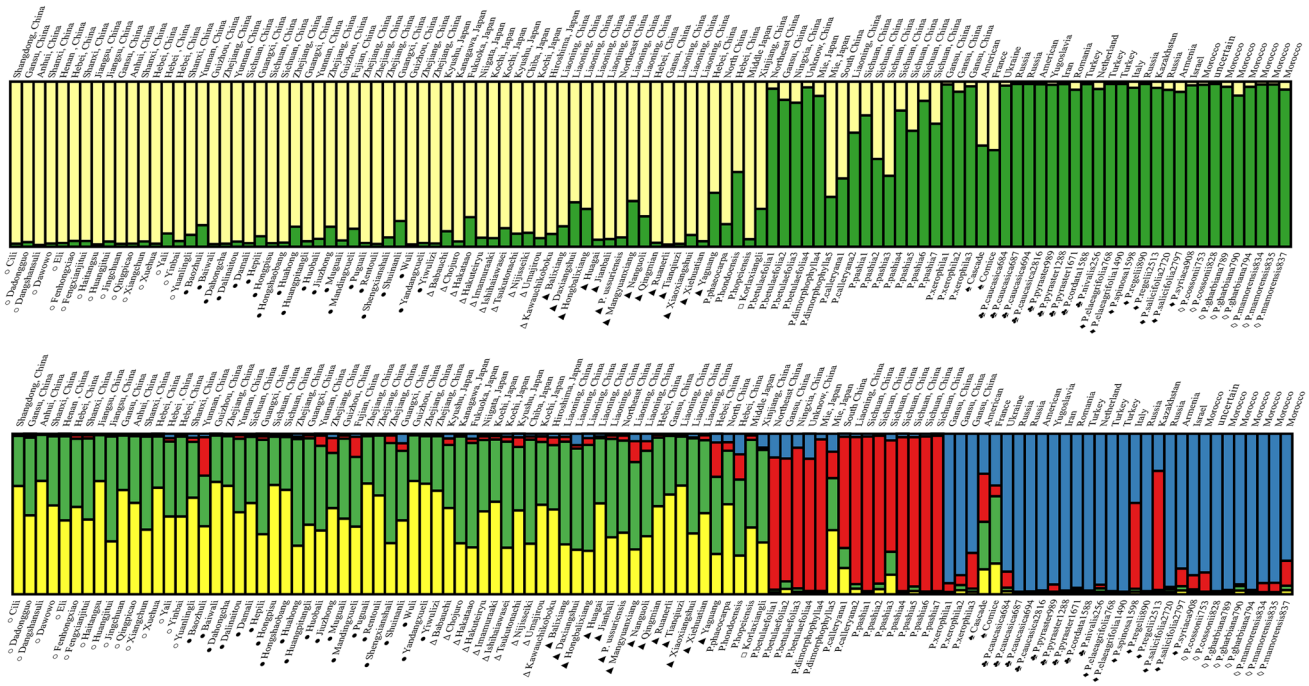

Fig. 3 Genetic relationships among the 110 accessions of *Pyrus* revealed by a Bayesian modeling approach under $K = 2$ (*top*) and $K = 4$ (*bottom*)

number of *Pyrus* species, divided *Pyrus* into Oriental pears and Occidental pears based on AFLP, RAPD, and SSR markers. These results support the traditional view that the genus *Pyrus* consists of two geographic species groups: Oriental pears and Occidental pears.

Four wild Oriental pea pear species composed subgroup III and subgroup IV in the UPGMA dendrogram (Fig. 4). *P. dimorphophylla*, *P. calleryana*, and *P. pashia* were clustered together in subgroup III. Four accessions of *P. betulaefolia* from different places clustered together in subgroup IV and were located between Occidental pears and other Oriental pears, which is consistent with the results obtained from RAPD markers (Teng et al. 2002) and AFLP markers (Bao et al. 2008). In the model with two genepools in STRUCTURE (Fig. 3), we noticed that the green cluster mainly contained Occidental pears and two wild Asian species, *P. xerophila* and *P. betulaefolia*, native to North China (Yu 1979). These results indicated that the Occidental pear species' cluster was linked to the Oriental pear cluster through *P. betulaefolia*, as had been proposed by Challice and Westwood (1973). However, in the model with four genepools in STRUCTURE (Fig. 3), *P. dimorphophylla*, *P. calleryana, P. pashia*, and *P. betulaefolia* were allotted predominantly to one cluster, inferring their relatively close relationship. Two accessions in *P. dimorphophylla* appeared to be of different origins, *P. dimorphophylla* 5 with four genepools and *P. dimorphophylla* 4 with two major genepools, suggesting *P. dimorphophylla* had a complex genetic background.

Pyrus xerophila is a wild Oriental pear mainly found in Gansu Province, China, where cultivars of *P. communis* have been introduced through the Silk Road and cultivated for a long time (Teng and Tanabe 2004). In a previous study, we proposed that *P. xerophila* might be an ancient genetic recombinant that arose by interspecies hybridization involving Oriental and Occidental species based on the analysis of *Adh* and *LEAFY* sequences (Zheng et al. 2011). In the results of the RAPD markers (Teng et al. 2001), *P. xerophila* clustered loosely with 'Beijing Baili', a famous *P. ussuriensis* cultivar and shared several common RAPD bands with the Occidental pears, implying some relationship between *P. xerophila* and *P. ussuriensis*, as well as the Occidental pears. In this study, STRUCTURE results ($K = 4$) (Fig. 3) showed that *P. xerophila* was composed of one major genepool that mostly appeared in Occidental pears, and the UPGMA dendrogram (Fig. 4) also indicated that *P. xerophila* was clustered with the Occidental pears, which further confirmed their close genetic relationship. However, a more extensive sampling of *P. xerophila* should be carried out to elucidate the origin of *P. xerophila*.

Pyrus hondoensis, native to Japan, was once classified as a variety of *P. ussuriensis*. In the UPGMA dendrogram, *P. hondoensis* and two *P. ussuriensis* cultivars, 'Mangyuanxiang' and 'Yaguang', were clustered together in subgroup I,

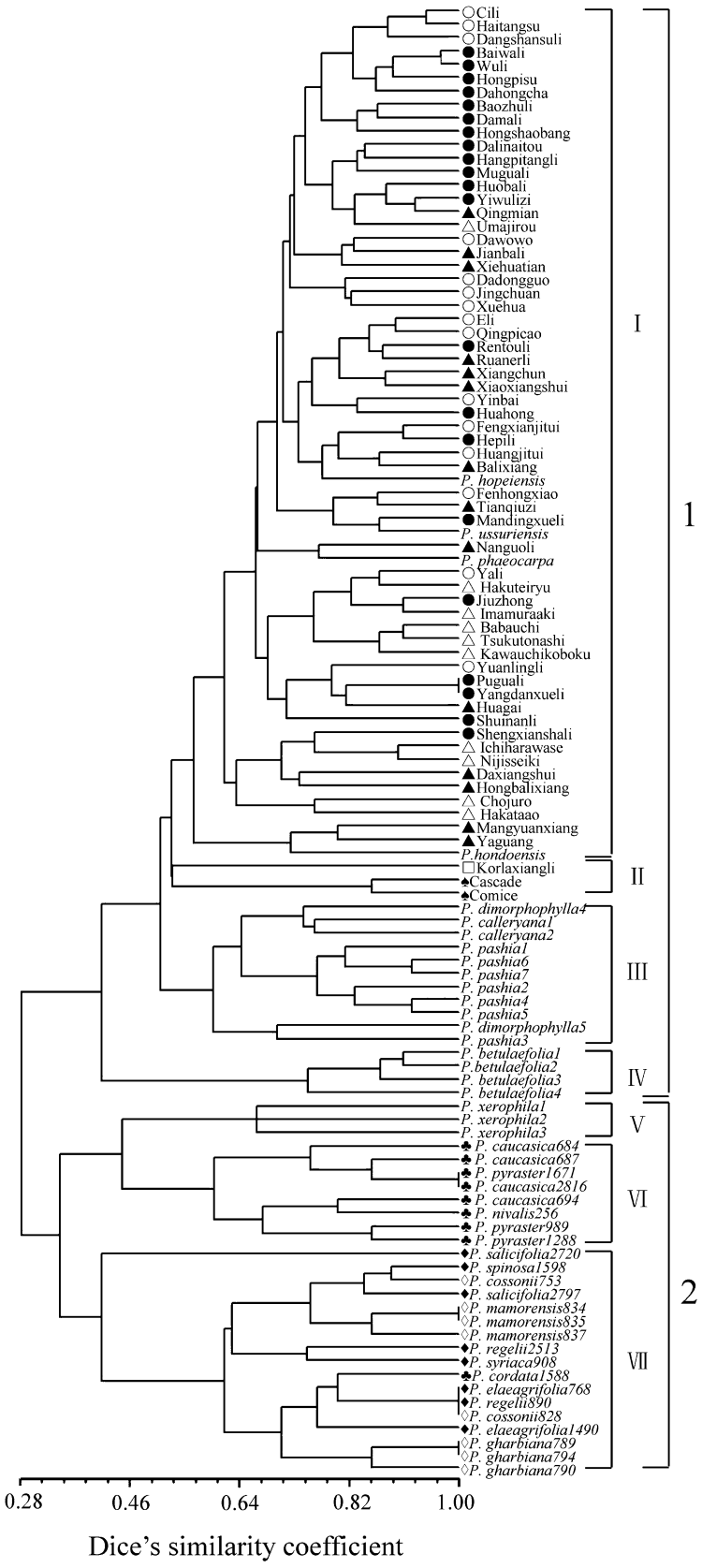

Fig. 4 Dendrogram of 110 pear species and cultivars based on their genetic similarity coefficients. UPGMA cluster analysis based on Dice's similarity coefficients (Nei and Li, 1979) was used to generate the dendrogram. '○': *P. pyrifolia* Chinese white pear group; '●': *P. pyrifolia*; '△': Japanese pear; '▲': *P. ussuriensis*; '□': *P. sinkiangensis*; '♠': *P. communis*; '♣': Species originating from Europe; '♦': Species originating from West Asia; and '◊': Species originating from North Africa

which supports our previous results with RAPD, AFLP, and SSR markers (Teng et al. 2002; Bao et al. 2007, 2008).

All of the Occidental pear accessions, except 'Cascade' and 'Comice', were clustered into two subgroups (Fig. 4). Accessions in Subgroup VI belonged to the European species, except for *P. cordata*, which occurs in the western margin of Europe, southwestern England, western France, and north-west of the Iberian Peninsula (Challice and Westwood 1973). *P. cordata* 1588 clustered with species from West Asia and North Africa in subgroup VII and was far from the other European species (Fig. 4), but it has a similar genetic background to some of the European species ($K = 4$, Fig. 3), which supports the view that *P. cordata* is a relict species linked to North African, West Asian, and East Asian species (Challice and Westwood 1973; Aldasoro et al. 1996). *P. caucasica* and *P. pyraster* were reported to have very similar botanical characteristics (Challice and Westwood 1973), and they were once treated as a variety of *P. communis*. In this study, accessions from *P. caucasica* and *P. pyraster* appeared in two subclades of subgroup VI, indicating that they have a close relationships with each other. Three of four accessions in *P. caucasica* were clustered with *P. pyraster* 1671 in one subclade, while *P. caucasica* 694 was with the other accessions of *P. pyraster*.

Subgroup VII consisted of pear accessions from West Asia and North Africa. In the results of STRUCTURE ($K = 4$), '*P. spinosa* 1598' and '*P. regelii* 2513' appeared to be of an admixed origin. The tomentose indumentum on both sides of the young leaves was a common feature in *P. spinosa* and was usually lost in the mature leaves in summer, which is very similar to those of *P. betulaefolia*. *P. spinosa* might be of a hybrid origin involving wild Asian pears, probably *P. betulaefolia*. Two accessions of *P. regelii*, '*P. regelii* 2513' with two genepools and '*P. regelii* 890' with one genepool were shown to be of different origins. More samples are needed for further studies. *P. salicifolia* was thought to be a synonym for *P. nivalis* or a hybrid of *P. nivalis* and *P. communis* (Aldasoro et al. 1996). However, in the UPGMA results, two accessions of *P. salicifolia* did not cluster with *P. nivalis* but grouped with species from West Asia and North Africa (Fig. 4). In the case of *P. cossonii*, native to North Africa, one genotype clustered with '*P. spinosa* 1598' and *P. salicifolia* 2797, and the other grouped with '*P. regelii* 890' and '*P. elaeagrifolia* 768', which reflected its complex genetic background. Another North African species, *P. mamorensis*, was shown by STRUCTURE ($K = 4$) analysis (Fig. 4) to have at least two genepools, of which one genepool (red) mostly appeared in Oriental wild pears, suggesting that this species was more related to the Oriental wild species as indicated by Challice and Westwood (1973).

Hybrid origins of cultivars

Although cultivars of *P. ussuriensis* differed morphologically from other cultivated pears native to East Asia, all accessions from *P. pyrifolia* Chinese white pear group, *P. pyrifolia*, and *P. ussuriensis* were clustered together into subgroup I in the UPGMA dendrogram, and the STRUCTURE analysis also showed that their genotypes were assigned as admixture of two genepools (Fig. 3, $K = 4$). The results in this study were different from our previous results with RAPD (Teng et al. 2002) and SSR markers (Bao et al. 2007), where the cultivars from *P. ussuriensis* clustered independently from other species' accessions. Iketani et al. (2012) reported that *P. pyrifolia* Chinese white pear group and *P. ussuriensis* from China also could not be distinguished strictly by SSR markers. In *Pyrus*, interspecific hybridization has been considered the major mode of evolution (Iketani et al. 2012; Zheng et al. 2008, 2011, 2014). *P. pyrifolia* Chinese white pear group and *P. ussuriensis* are extensively cultivated in North China. There was no reproductive barrier among these species and horizontal gene transfer may cause their poor resolution in the phylogenetic tree. Introgressive gene flow and hybrid speciation might be expected to have a higher prevalence between closely related species and populations. The STRUCTURE analysis ($K = 4$) showed some accessions in *P. pyrifolia* with three major genepools, of which one genepool (red) appeared in the Oriental wild species, such as 'Baozhuli'; 'Huobali'; 'Jiuzhong'; and 'Mandingxueli', suggesting that these accessions might be of a hybrid origin involved with Oriental wild species. Four *P. ussuriensis* accessions ('Daxiangshui', 'Mangyuanxiang', 'Nanguoli', and 'Yaguang') also appeared to have three or four genepools, inferring their hybridized origin. These results confirmed that hybridization extensively existed among cultivars of *P. pyrifolia* Chinese white pear group, *P. pyrifolia* and *P. ussuriensis*.

'Korlaxiangli', a famous cultivar of *P. sinkiangensis*, was clustered with two cultivars of *P. communis*, 'Cascade' and 'Comice', in subgroup II in the UPGMA dendrogram, showing the genetic background of *P. communis*. Our previous study showed that *P. sinkiangensis* is of hybrid origin and at least *P. communis*, *P. armeniacaefolia*, and Chinese white pears or sand pears have been involved (Teng et al. 2001). 'Cascade' was reported to be a hybrid between 'Max Red Bartlett' and 'Comice' (NCGR-Corvallis *Pyrus* Germplasm, http://www.ars.usda.gov/main//Docs.htm?docid=11372). The results of STRUCTURE also showed that these two cultivars displayed an admixed genepool, which inferred that 'Comice' and 'Cascade' might be of a hybrid origin involved with Asian pears.

Synonymous cultivars or accessions

In the Oriental group, two *P. pyrifolia* cultivars, 'Puguali' and 'Yangdanxueli', showed 100 % similarity in the UPGMA dendrogram and STRUCTURE results. Among all 24 primers used in this study, 15 primers amplified the same fragments in these two cultivars (Supplementary data 2). All of the results suggested that these two cultivars might be synonyms. In the Occidental group, *P. elaeagrifolia* 768 from Turkey, *P. regelii* 890 from Russia, and *P. cossonii* 828 showed 100 % similarity. A similar situation was also observed between *P. pyraster* 1671 from Romania and *P. caucasica* 2816 from Armenia. Because only a few primers amplified successfully in these Occidental accessions, we could not determine if they were synonyms. More RBIP makers will be needed to confirm their relationships. *P. mamorensis* 834 and 835, and *P. gharbiana* 789 and 794, all from Morocco showed 100 % similarity, and might be treated as the same genotype.

Conclusions

In summary, the present study predicted retrotransposons in *Pyrus* and provided a new approach to develop RBIP markers. Ten LTR retrotransposon subfamilies in pear were identified and the *Ppcr1* retrotransposon was proven to be duplicated many times in the development of cultivated Asian pears. Results inferred from the data of RBIP markers confirmed that *Pyrus* could be divided into Occidental and Oriental groups, and that extensive hybridization events occurred during the development of Asian pear cultivars. *P. xerophila*, which is native to Gansu Province, China, was proven to be an interspecies hybrid involving Oriental and Occidental species. Some Occidental pear cultivars or species, such as 'Cascade', 'Comice', and '*P. spinosa* 1598', were observed to be interspecific hybrids between Occidental and Oriental species, and some accessions, such as 'Puguali' and 'Yangdanxueli', were identified as synonyms. New findings in this study will be important to further understand the phylogeny of *Pyrus*. More RBIP markers will be needed to produce a comprehensive understanding of the complex relationships and evolution in the *Pyrus* species.

Acknowledgments This work was financed by a Grant from the National Natural Science Foundation of China (No. 31201592), a Specialized Research Fund for the Doctoral Program of Higher Education (20110101110091), and a Grant for Innovative Research Team of Zhejiang Province of China (2013TD05).

References

Aldasoro JJ, Aedo C, Garmendia FM (1996) The genus *Pyrus* L. (Rosaceae) in south-west Europe and North Africa. Bot J Linn Soc 121(2):143–158

Bailey LH (1917) *Pyrus*. Standard cyclopedia of horticulture, vol V. Macmillan, New York, pp 2865–2878

Bao L, Chen K, Zhang D, Cao Y, Yamamoto T, Teng Y (2007) Genetic diversity and similarity of pear (*Pyrus* L.) cultivars native to East Asia revealed by SSR (simple sequence repeat) markers. Genet Resour Crop Evol 54(5):959–971

Bao L, Chen K, Zhang D, Li X, Teng Y (2008) An assessment of genetic variability and relationships within Asian pears based on AFLP (amplified fragment length polymorphism) markers. Sci Hortic 116(4):374–380

Bassil N, Postman JD (2010) Identification of European and Asian pears using EST-SSRs from *Pyrus*. Genet Resour Crop Evol 57(3):357–370

Baucom RS, Estill JC, Leebens-Mack J, Bennetzen JL (2009) Natural selection on gene function drives the evolution of LTR retrotransposon families in the rice genome. Genome Res 19(2):243–254

Bergman CM, Quesneville H (2007) Discovering and detecting transposable elements in genome sequences. Brief Bioinform 8(6):382–392

Cavallini A, Natali L, Zuccolo A, Giordani T, Jurman I, Ferrillo V, Vitacolonna N, Sarri V, Cattonaro F, Ceccarelli M, Cionini PG, Morgante M (2010) Analysis of transposons and repeat composition of the sunflower (*Helianthus annuus* L.) genome. Theor Appl Genet 120(3):491–508

Challice JS, Westwood MN (1973) Numerical taxonomic studies of the genus *Pyrus* using both chemical and botanical characters. Bot J Linn Soc 67(2):121–148

Cossu RM, Buti M, Giordani T, Natali L, Cavallini A (2012) A computational study of the dynamics of LTR retrotransposons in the *Populus trichocarpa* genome. Tree Genet Genomes 8(1):61–75

Diwan N, Cregan PB (1997) Automated sizing of fluorescent-labeled simple sequence repeat (SSR) markers to assay genetic variation in soybean. Theor Appl Genet 95(5–6):723–733

Dondoshansky I, Wolf Y (2002) Blastclust (NCBI Software Development Toolkit). NCBI, Bethesda

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Earl DA, VonHoldt BM (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv Genet Resour 4(2):359–361

Ellinghaus D, Kurtz S, Willhoeft U (2008) LTRharvest, an efficient and flexible software for de novo detection of LTR retrotransposons. BMC Bioinform 9(1):18

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14(8):2611–2620

Fan L, Zhang M, Liu Q, Li L, Song Y, Wang L, Zhang S, Wu J (2013) Transferability of newly developed pear SSR markers to other Rosaceae Species. Plant Mol Biol Rep 31(6):1271–1282

Flavell AJ, Dunbar E, Anderson R, Pearce SR, Hartley R, Kumar A (1992) Ty1-*copia* group retrotransposons are ubiquitous and heterogeneous in higher plants. Nucleic Acids Res 20(14):3639–3644

Havecker ER, Gao X, Voytas DF (2004) The diversity of LTR retrotransposons. Genome Biol 5(6):225

Iketani H, Katayama H, Uematsu C, Mase N, Sato Y, Yamamoto T (2012) Genetic structure of East Asian cultivated pears (*Pyrus* spp.) and their reclassification in accordance with the nomenclature of cultivated plants. Plant Syst Evol 298(9):1689–1700

Jakobsson M, Rosenberg NA (2007) CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics 23(4):1801–1806

Jing R, Knox MR, Lee JM, Vershinin AV, Ambrose M, Ellis TN, Flavell AJ (2005) Insertional polymorphism and antiquity of

PDR1 retrotransposon insertions in *Pisum* species. Genetics 171(2):741–752
Kalendar R, Schulman AH (2006) IRAP and REMAP for retrotransposon-based genotyping and fingerprinting. Nat Protoc 1(5):2478–2484
Kalendar R, Flavell AJ, Ellis TH, Sjakste T, Moisy C, Schulman AH (2011) Analysis of plant diversity with retrotransposon-based molecular markers. Heredity (Edinb) 106(4):520–530
Kim H, Yamamoto M, Hosaka F, Terakami S, Nishitani C, Sawamura Y, Yamane H, Wu JZ, Matsumoto T, Matsuyama T, Yamamoto T (2011) Molecular characterization of novel Ty1-*copia*-like retrotransposons in pear (*Pyrus pyrifolia*). Tree Genet Genomes 7(4):845–856
Kim H, Terakami S, Nishitani C, Kurita K, Kanamori H, Katayose Y, Sawamura Y, Saito T, Yamamoto T (2012) Development of cultivar-specific DNA markers based on retrotransposon-based insertional polymorphism in Japanese pear. Breed Sci 62(1):53–62
Kohany O, Gentles AJ, Hankus L, Jurka J (2006) Annotation, submission and screening of repetitive elements in Repbase: RepbaseSubmitter and Censor. BMC Bioinform 7:474
Koressaar T, Remm M (2007) Enhancements and modifications of primer design program Primer3. Bioinformatics 23(10):1289–1291
Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and clustal X version 2.0. Bioinformatics 23(21):2947–2948
McNeill J (2012) International Code of Nomenclature for algae, fungi and plants (Melbourne Code). Koeltz Scientific Books, Germany
Monte-Corvo L, Cabrita L, Oliveira C, Leitao J (2000) Assessment of genetic relationships among *Pyrus* species and cultivars using AFLP and RAPD markers. Genet Resour Crop Evol 47(3):257–265
Nei M, Li W (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc Natl Acad Sci USA 76(10):5269–5273
Oliveira CM, Mota M, Monte-Corvo L, Goulao L, Silva DM (1999) Molecular typing of *Pyrus* based on RAPD markers. Sci Hortic 79(3):163–174
Peterson DG, Schulze SR, Sciara EB, Lee SA, Bowers JE, Nagel A, Jiang N, Tibbitts DC, Wessler SR, Paterson AH (2002) Integration of Cot analysis, DNA cloning, and high-throughput sequencing facilitates genome characterization and gene discovery. Genome Res 12(5):795–807
Potter D, Eriksson T, Evans RC, Oh S, Smedmark JEE, Morgan DR, Kerr M, Robertson KR, Arsenault M, Dickinson TA, Campbell CS (2007) Phylogeny and classification of Rosaceae. Plant Syst Evol 266(1–2):5–43
Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. Genetics 155(2):945–959
Rohlf FJ (1998) Numerical taxonomy and multivariate analysis system version 2.0. Exeter Publishing, Setauket
Rosenberg NA (2004) DISTRUCT: a program for the graphical display of population structure. Mol Ecol Notes 4(1):137–138
Sabot F, Schulman AH (2006) Parasitism and the retrotransposon life cycle in plants: a hitchhiker's guide to the genome. Heredity (Edinb) 97(6):381–388
SanMiguel P, Tikhonov A, Jin YK et al (1996) Nested retrotransposons in the intergenic regions of the *maize* genome. Science 274(5288):765–768
SanMiguel P, Gaut BS, Tikhonov A, Nakajima Y, Bennetzen JL (1998) The paleontology of intergene retrotransposons of *maize*. Nat Genet 20(1):43–45
Schnable PS, Ware D, Fulton RS et al (2009) The B73 *maize* genome: complexity, diversity, and dynamics. Science 326(5956):1112–1115
Smit AFA, Hubley R, Green P (1996–2004). RepeatMasker: Open-3.0. Published on the web. http://www.repeatmasker.org. Accessed 31 January 2014
Teng Y, Tanabe K (2004) Reconsideration on the origin of cultivated pears native to East Asia. Fourth Int Symp Taxon Cultiv Plants 634:175–182
Teng Y, Tanabe K, Tamura F, Itai A (2001) Genetic relationships of pear cultivars in Xinjiang, China, as measured by RAPD markers. J Hortic Sci Biotechnol 76(6):771–779
Teng Y, Tanabe K, Tamura F, Itai A (2002) Genetic relationships of *Pyrus* species and cultivars native to East Asia revealed by randomly amplified polymorphic DNA markers. J Am Soc Hortic Sci 127(2):262–270
Velasco R, Zharkikh A, Affourtit J et al (2010) The genome of the domesticated apple (*Malus x domestica* Borkh.). Nat Genet 42(10):833–839
Vicient CM, Kalendar R, Anamthawat-Jonsson K, Schulman AH (1999) Structure, functionality, and evolution of the *BARE*-1 retrotransposon of barley. Genetica 107(1–3):53–63
Waugh R, McLean K, Flavell AJ, Pearce SR, Kumar A, Thomas BB, Powell W (1997) Genetic distribution of *Bare*-1-like retrotransposable elements in the barley genome revealed by sequence-specific amplification polymorphisms (S-SAP). Mol Gen Genet 253(6):687–694
Wicker T, Sabot F, Hua-Van A et al (2007) A unified classification system for eukaryotic transposable elements. Nat Rev Genet 8(12):973–982
Wierdl M, Dominska M, Petes TD (1997) Microsatellite instability in yeast: dependence on the length of the microsatellite. Genetics 146(3):769–779
Wu J, Wang Z, Shi Z et al (2013) The genome of the pear (*Pyrus bretschneideri* Rehd.). Genome Res 23(2):396–408
Yamada NA, Smith GA, Castro A, Roques CN, Boyer JC, Farber RA (2002) Relative rates of insertion and deletion mutations in dinucleotide repeats of various lengths in mismatch repair proficient mouse and mismatch repair deficient human cells. Mutat Res Fund Mol M 499(2):213–225
Yamamoto T, Kimura T, Sawamura Y, Manabe T, Kotobuki K, Hayashi T, Ban Y, Matsuta N (2002) Simple sequence repeats for genetic analysis in pear. Euphytica 124(1):129–137
Yao L, Zheng X, Cai D, Gao Y, Wang K, Cao Y, Teng Y (2010) Exploitation of *Malus* EST-SSRs and the utility in evaluation of genetic diversity in *Malus* and *Pyrus*. Genet Resour Crop Evol 57(6):841–851
Yu T (1979) Taxonomy of the fruit tree in China. Agriculture Press, Beijing (in Chinese)
Zheng X, Cai D, Yao L, Teng Y (2008) Non-concerted ITS evolution, early origin and phylogenetic utility of ITS pseudogenes in *Pyrus*. Mol Phylogenet Evol 48(3):892–903
Zheng X, Hu C, Spooner D, Liu J, Cao J, Teng Y (2011) Molecular evolution of *Adh* and *LEAFY* and the phylogenetic utility of their introns in *Pyrus* (Rosaceae). BMC Evol Biol 11(1):255
Zheng X, Cai D, Potter D, Postman J, Liu J, Teng Y (2014) Phylogeny and evolutionary histories of Pyrus L. revealed by phylogenetic trees and networks based on data from multiple DNA sequences. Mol Phylogenet Evol. doi:10.1016/j.ympev.2014.07.009