Phylogeny of *Pyrus* and their relationships inferred from sequences of nuclear NIA- β intron and chloroplast *ndhC-trnV* and *trnR-atpA*

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Abstract

Background: Pear (*Pyrus* L.) belongs to subtribe Malinae, tribe Maleae, family Rosaceae. According to the geography distribution, it could be divided into Oriental pear and Occidental pear. Though the phylogeny of *Pyrus* was complicate, previous study referring to morphology, nuclear genes and chloroplast genes made the relationships clear gradually. However, they had lower sequence divergence and less information, therefore it hardly solved the phylogeny of *Pyrus*.

Results: A total of 100 accessions from Oriental and Occidental pears were used to elucidate the phylogeny of *Pyrus* by one nuclear NIA-i3 intron and two chloroplast regions (*ndhC-trnV* and *trnR-atpA*) with higher polymorphism. The Neighbor-Net phylogenetic network indicated that the phylogenetic relationships were complicate based on *ndhC-trnV* and *trnR-atpA*. Oriental pear and Occidental pear were separated in the tree of NIA-i3, *P. betulafolia*, *P. pashia* (except *P. pashia* 'P10-3'_1 and *P. pashia* 'P23-4'_1) were monophyly; several *P. ussuriensis* were closely related with *P. xerophila*. However, the phylogenetic relationships of Chinese White Pear, *P. pyrifolia* and some *P. ussuriensis* still could not be well solved; West Asian species and European species were mingled together. Occidental pear *P. caucasica* 684, *P. pyrastrer* 989, *P. elaeagrifolia* 2817 might be hybrids between Oriental pear and Occidental pear.

Conclusions: The phylogenetic relationships of *Pyrus* were still complicate because interspecies and intraspecies of Oriental pear and Occidental pear respectively were intercrossed. More genes and more accessions were needed to solve the phylogenetic relationships in *Pyrus* and to explore the possible parents.

Introduction

Pear (*Pyrus* L.) belongs to subtribe Malinae, tribe Maleae, family Rosaceae (McNeill et al., 2012; Potter et al., 2007; Teng et al., 2017). It originated from southwest of China and widely distributed in many countries around the world, such as American, Japan, Korean, New Zealand, Italy, etc. According to the geography distribution, it could be divided into Oriental pear and Occidental pear. Oriental pear was mainly from China, which 13 species were approved in China by Yu (Yu et al., 1979), including *P. xbrechtsneideri* Rehd., *P. pyrifolia* (Burm.) Nak., *P. ussuriensis* Maxim., *P. xsinkiangensis*, *P. betulaeifolia* Bge., *P. calleryana*, *P. pashia* D.Don, *P. phaeocarpa* Rehd., *P. xserulata* Rehd., *P. pseudopashia* Yu, *P. xhopeiensis* Yu, *P. xerophila* Yu, *P. armeniacaefolia* Yu. While Occidental pear was mainly from Europe, North Africa, Central Asia, Iran and so on, including *P. communis* L., *P. amygdaliformis* Vill., *P. elaeagrifolia* Pall., *P. salicifolia* Pall., *P. Syriaca* Boiss., *P. regelii* Rehd., *P. nivalis* Jacq., *P. cordata* Desv., etc.

Though the phylogeny of *Pyrus* was complicate, previous study referred to morphology, nuclear genes and chloroplast genes even the genome resequencing (Jiang et al., 2016; Jiang et al., 2015; Wu et al., 2018; Yue et al., 2018; Zheng et al., 2011, 2014) made the relationships clear gradually. The results from different molecular markers indicated that Chinese White Pear (*P. xbrechtsneideri*), Chinese Sand Pear (*P. pyrifolia*) and Japanese pear cultivars had close relationships to one another, but might with introgression of different species (Jiang et al., 2016; Yue et al., 2018). Therefore, they were suggested to be nominated *Pyrus pyrifolia* White Pear Group (Teng and Tanabe, 2004), *Pyrus pyrifolia* Sand Pear Group and *Pyrus pyrifolia* Japanese Pear Group (Teng, 2017), respectively. Cultivated *P. ussuriensis* was approved to be hybrids from wild *P. ussuriensis* and Chinese White Pear (Yu et al., 2016). Cultivars in *P. ussuriensis* might be maternal inheritance of cultivars in *P. pyrifolia* (Yue et al., 2018). The genetic diversity of *P. betulaeifolia* in northern China was divided by Taihang Mountains (Zong et al., 2017). *P. calleryana* and *P. pashia* might have common and recent ancestor because of the low divergence in chloroplast (Liu et al., 2013).

However, these studies have been limited by using only chloroplast information or nuclear information or few species, and the phylogeny of *Pyrus* and their relationships still need to be fully addressed. Therefore, both nuclear genes and chloroplast genes were considered in our study. Chloroplast genes were generally used in the phylogeny and evolution analysis of higher categories of plants because of lower evolution rate (Byrne et al., 2012; Katayama et al., 2012; Terakami et al., 2012; Wuyun et al., 2013). In previous studies, *ndhC-trnV* was one of the five most promising regions for phylogenetic analysis in terms of variability (Byrne et al., 2012). Lin et al. (2015) explored 27 cpDNA regions to evaluate molecular markers for distinguishing *Phalaenopsis* species, of which 15 cpDNA markers were highly variable among moth orchids, with polymorphic information contents 8.5 in *trnR-atpA*. Six non-coding regions of chloroplast were used to construct the phylogeny of eight pear varieties from five *Pyrus* species (Kimura et al., 2003). Three Asian pear groups and one European pear group could be identified with the former intraspecific and interspecific diversities.

NIA gene contained four exons and three introns and it might be single copy, or two or more copies (Harris et al., 2000; Salanoubat & Ha, 1993; Howarth and Baum, 2002; Yi et al., 2007, 2008; Vaucheret et al. 1989). Zhou & Kleinof (1996) thought it could be used in the phylogenetic study of species with distant relationships. Later, Howarth & Baum (2002) considered that it suitable for phylogenetic study of species with recent divergence due to faster evolutionary rate of intron. They found that the sequence differences of NIA-i3 were 5.4 that of ITS, and now it had been widely used in *Paulownia* (Howarth & Baum, 2002), *Betula* (Li et al., 2007), *Elaeagnus angustifolia* (Douglas, 2007), *Solanum* (Rodríguez et al., 2009).

The present research is an outgrowth of our prior phylogenetic studies of *Pyrus* (Zheng et al., 2014). In this research, more accessions from Oriental pear and Occidental pear, and two chloroplast regions (*ndhC-trnV* & *trnR-atpA*) and one nuclear gene NIA-i3 with higher polymorphism were used, to elucidate the complicate phylogeny of *Pyrus*.

Materials And Methods

2.1 Leaflet collection and DNA extraction

A total of 100 accessions from 13 species of Oriental pear and 12 species of Occidental species were used in our research (Table 1), of which the accessions of *P. calleryana*, *P. pashia*, *P. betulaeifolia* were from previous studies having different haplotypes (Liu et al., 2012, 2013; Zheng et al., 2014; Zong et al., 2014),

with *Malus domestica* Borkh., red apple as out groups. Leaflets were collected in the early spring and stored in -80°C. DNAs were extracted by modified SDS methods (Teng et al., 2001).

2.2 PCR amplification and sequencing

PCR amplifications were carried out in a total volume of 40 µl for two cpDNA fragments (*ndhC-trnV* & *trnR-atpA*). For *ndhC-trnV*: 10×buffer, 0.2 mM of Mg²⁺, 0.2mM of dNTPs, 0.5pM of each primer, 2Unit of Taq (Table 2). Cycling conditions were 94 °C for 4min followed by 35 cycles at 94 °C for 30s, 55 °C for 30s, 67 °C for 1min, and a final extension at 72 °C for 7min. For *trnR-atpA*: 1×buffer, 0.25mM of Mg²⁺, 0.2mM of dNTPs, 0.25pM of each primer, 1Unit of Taq. Cycling conditions were 94 °C for 4min followed by 35 cycles at 94 °C for 30s, 55 °C for 30s, 70 °C for 1min, and a final extension at 72 °C for 7min.

The primer sequences of NIA were used as Howarth & Baum (2002) mentioned (Table 2). PCR amplification in a total volume of 25 µl were as follows: 10×buffer, 3.0 mM of Mg²⁺, 0.2mM of dNTPs, 0.4µM of each primer, 0.625 U of Taq. Cycling conditions were 94 °C for 4min followed by 35 cycles at 94 °C for 60s, 58 °C for 60s, 72 °C for 1min, and a final extension at 72 °C for 7min.

2.3 Sequence alignment and model selection

Sequences were edited by Staden package 2.0.0b11 (Staden, 1996) and aligned in CLUSTALX 2.1 (Larkin et al., 2007), with further manual alignments by Mesquite 3.61 (Maddison & Maddison, 2019). The molecular substitution models were evaluated by jModelTest (Darriba et al., 2012) to select the preferred model that could be used in *BEAST (Drummond & Rambaut, 2007), separately for each cpDNA fragment. We used a likelihood ratio test to compare nested models, with a forward step-wise approach. For both loci, the selected model accounted for rate variation with a gamma-distributed rate variation among sites (Γ).

2.5 Phylogenetic analysis

Maximum parsimony (MP) analyses of two cpDNA regions and NIA-i3 were inferred by PAUP 4.0b10 (Swofford 2002). Settings were as follows: heuristic search, add 100000 repeats randomly, Tree-Bisection-Reconnection (TBR). The method g1 and ILD were used for testing parsimony signal and identity for three databases constructed by two cpDNA regions and NIA-i3, respectively (Farris et al., 1994).

Maximum likelihood (ML) analyses were performed by RaxML (Stamatakis, 2014) using the basic general time reversible model (GTR), 1000 alternative runs on distinct starting trees, and rapid bootstrapping with consensus generation. The ML trees with bootstrap value of 100 repeats sampling and best score were retrieved. Both MP and ML trees were run in Paup 4.0b10.

Likelihood value of each gene regions were calculated by jModelTest2, and the best model was selected by comparing likelihood values of different models. MrBayes analyses were run in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) under best model GTR. Settings were as follows: *Malus* as outgroup, MCMC (Markov Chain Monte Carlo), 3 runs with four chains for 10 million generations and using a burn-in fraction of 25%. The program was stopped when standard deviation value reached 0.001.

2.4 Allele choosing and phylogenetic tree reconstruction

Because of cloning sequencing of NIA-i3, more sequences were gained for every species. RAXML was used to analyze NIA sequence files (no gap included), genes with the minor allelic variants were used for final sequence analysis (Stamatakis et al., 2014). When choosing allelic, if multi-cloning sequences from the same species were in the same clade, the sequence closed to the tree base were chosen. Finally, new sequence data were obtained and used to construct NJ or ML or Bayesian tree.

Results And Discussion

3.1 The haplotypes and the nucleotide polymorphisms of two cpDNAs intergenic regions *ndhC-trnV* and *trnR-atpA*

The analysis of haplotypes and the nucleotide polymorphisms in two cpDNAs intergenic regions indicated that the haplotype number (H) of *ndhC-trnV* and *trnR-atpA* were 17 and 20, respectively (Table 3). The haplotype polymorphism (Hd) was 0.752 and 0.720, respectively; nucleotide polymorphisms (Pi) were 0.00325 and 0.00923, respectively. Tajima test (D) values were -1.88041 and -1.53301, both of which were negative, suggesting the evolution mode of sequences was negative selection. The P value of *ndhC-trnV* was P<0.05, indicated the significant differentiation between the number of segregative sites and the value of nucleotide polymorphisms in each sequences, and this intergenic region did not follow the neutral model during evolution. While the P value of *trnR-atpA* was P>0.10, indicated no significant difference between the number of segregative sites and the value of nucleotide polymorphisms in each sequences, and this intergenic region followed the neutral model during evolution.

Without considering missing/gaps and identical sites, the haplotype number of *ndhC-trnV* was 17. The haplotypes in Oriental pear were cH2-cH7, cH17 (cH stands for *ndhC-trnV* haplotype), and the main haplotype was cH2 and cH4 (table 4). The richest haplotypes were in *P. pyrifolia* with four haplotypes, maybe because the accessions were originated from different places, such as Zhejiang, Fujian, Japan, and so on, of which the different environment led to evolutions of them. Both *P. bretschneideri* and *P. ussuriensis* had two haplotypes of cH2 and cH4. *P. betulafolia*, *P. calleryana* and *P. pashia* has 1, 2, 3 haplotypes, respectively. The main haplotype of three of them was cH2. Though different haplotypes from wide populations were chosen as representative samples, the haplotypes were not rich for them in this study. There might be not too much divergence during the evolution of these two intergenic regions in chloroplast, and might also because Indels were not considered. The haplotypes in Occidental pear were cH8-cH16, and the main haplotype was cH8. There were two haplotypes in *P. pyraeaster*, *P. cordata*, *P. nivalis*, *P. elaeagnifolia*, *P. salicifolia* and *P. syriaca*, and only one haplotype in the rest species of Occidental pear, might

because of limited accessions in each species. Moreover, *P. caucasica* 684 had cH4 haplotype which was one of the main haplotype in Oriental pear. Pears from Pakistan had haplotypes of cH5, cH8, cH15 and cH16. 'bali 1' was proposed to be Oriental pear, while 'bali 2', 'bali 3', 'bali 4' might be Occidental pear, but the species of them were still unknown.

Without considering missing/gaps and identical sites, the haplotype number of *trnR-atpA* was 19, of which rH1 (rH stands for *trnR-atpA* haplotype) was the haplotype of red flesh apple from *Malus*. There were six haplotypes in Oriental pear, which were rH3-rH6, rH8, rH18, and the main haplotype was rH5 (table 5). Haplotype in most of Oriental pears was simple, for example, *P. bretschneideri*, *P. pyrifolia*, *P. ussuriensis* and *P. calleryana* had only one haplotype of rH3, while *P. pashia* and *P. betulifolia* had two haplotypes, respectively.

There were 13 haplotypes in Occidental pear, which consisted of rH2, rH7-rH20 except rH8 and rH18, and the main haplotype was rH2 and rH11. The numerical order of haplotypes was: *P. elaeagnifolia* (four) > *P. nivalis* & *P. spinosa* & *P. mamorensis* (three) > *P. communis* & *P. pyrastrer* & *P. cordata* (two) > *P. regelii* & *P. syriaca* & *P. gharbiana* (one), which had richer haplotypes than Oriental pear. Three pear cultivars from Pakistan had haplotypes of rH2, and only one had rH6 haplotype. In the NN network, they had closer relationships with Oriental pears (Figure 3); *P. caucasica* 684 still had the haplotype of rH18 which was only in Oriental pear. Combined the results from *ndhC-trnV* above, the female parent of *P. caucasica* 684 might be from Oriental pear.

Without considering missing/gaps, the number of haplotypes in *ndhC-trnV* and *trnR-atpA* were 87 and 64, respectively, which suggested too much Indels between these two intergenic regions. Besides, there were too many A, T, or polyA, polyT structure in these two intergenic regions, which bring difficulty to PCR amplification and sequencing.

3.2 Sequence comparison between two cpDNA intergenic regions and NIA

cpDNA had the characteristics of maternal inheritance, therefore there was only one sequence after amplification. The aligned sequence length of two intergenic regions from cpDNA (*ndhC-trnV* and *trnR-atpA*) was 1066 and 1213 sites (including gaps); the number of parsimony informative sites was 94 (8.8%) and 36 (3.0%); the number of coding Indel sites was 121 and 120, respectively (Table 6).

NIA was one of low-copy nuclear genes, and one or two sequences were obtained after PCR amplification in *Pyrus* (Figure 4). The aligned sequence length of NIA was 2118 sites (including gaps); the number of parsimony informative sites was 435 (20.5%); the number of coding Indel sites was 108 (Table 4). All sequences were submitted to GenBank, and the phylogenetic trees were saved in TreeBase (www.treebase.org).

In the sequences of two intergenic regions from cpDNA and NIA, the best models for *ndhC-trnV* and *trnR-atpA* were F81+G, HKY, while NIA-i3 was TPM2uf+G (Table 4). These models were used in the following analysis.

3.3 The Neighbor-Net phylogenetic network analysis of two intergenic regions from cpDNA

3.3.1 The Neighbor-Net phylogenetic network analysis of *ndhC-trnV*

The phylogenetic trees constructed with *ndhC-trnV* by Maximum Likelihood method did not separate the Oriental pear from Occidental pear obviously (Figure was not shown), therefore Neighbor-Net phylogenetic network was constructed based on uncorrected-P genetic distance. The results were more complicated than the previous results (Zheng et al., 2014), which several splits cross connected to form a complicated network (different color showed in Figure 3a). Most accessions were connected by red splits, eg. fifty-five accessions with main haplotype of cH4 in Oriental pear, 'bali 1' with cH5 haplotype; thirty-nine accessions with main haplotype of cH8 in Occidental pear, 'bali 2' (cH16), 'bali 4' with cH15, *P. salicifolia* 869 (cH11), *P. cossonii* 828(cH12), *P. cossonii* 829 (cH12). *P. pashia* P5-11 (cH6), *P. pashia* P20-3 (cH7) in Oriental pear, and *P. nivalis* 1590 (cH9), *P. elaeagnifolia* (cH9), *P. communis* 'cascade' (cH10), *P. mamorensis* (cH1, cH13), *Ppyrastrer* (cH14) in Occidental pear, and outgroup *Malus* (cH1) were mainly connected by green and purple splits. *P. mamorensis* had the same haplotype with outgroup, suggested that it might be more primitive species. Moreover, in the NN network based on Neighbor Joining tree (Figure 3b), cH6, cH7, cH1, cH9, cH10 and cH14 were connected between cH4 and cH8 which were main haplotypes of Oriental pear and Occidental pear, indicated the accessions with these haplotypes might be transitional species between Oriental pear and Occidental pear.

3.3.2 The Neighbor-Net phylogenetic network analysis of *trnR-atpA*

The phylogenetic trees constructed with *trnR-atpA* by Maximum Likelihood method separated the Oriental pear from Occidental pear obviously (Figure was not shown), but there was not too much differences among each species, thus the phylogenetic relationships among them were not well solved. Neighbor-Net phylogenetic network was constructed for *trnR-atpA*, and the results were more complicated than that of *ndhC-trnV* (Figure 4). The results showed that the relationships of interspecies and intraspecies in Oriental pear from Occidental pear were complex, and *Malus* was closer with Occidental pear than Oriental pear. *P. dimorphophylla* 6, 'bali 1', *P. caucasica* 687, *P. salicifolia* (rH15) were connected with red splits. *P. pyrifolia* 'Rentouli' (rH4) and *P. regelii* 890 (rH19) were connected by blue splits. rH18 (*P. caucasica* 684 and *P. betulifolia* BT1'), rH7 (*P. pyrastrer* 881, *P. spinosa* 1608), rH17 (*P. nivalis* 256) and rH2 were linked indirectly by orange splits. rH8 (*P. pashia* P20-3) and rH16 (*P. elaeagnifolia* 2482) were joined together by green splits. rH10 (*P. cordata* 750) and Oriental pears were linked by aqua blue. Furthermore, in the NN network based on Neighbor Joining tree (Figure 4b), *P. pashia* P20-3 (rH8), *P. caucasica* 684 (rH18), *P. betulifolia* BT1' (rH18), *P. elaeagnifolia* 1482 (rH16), *P. regelii* 890 (rH19), *P. elaeagnifolia* 1490 (rH9) were in between the main haplotypes of Oriental pear from Occidental pear, to further suggest that *P. pashia*, *P. betulifolia*, and *P. regelii* might be transitional species or relic species.

3.4 The phylogenetic analysis of NIA-i3

3.4.1 The phylogenetic trees of *Pyrus* constructed by NIA-i3

The preliminary experiment showed that NIA-i3 had two copies in both *Malus* and *Pyrus*. These two copies might be appeared before these two species evolved, latterly, Indels and mutation led to the differentiation of species during evolution. After clone and sequencing, one to five NIA-i3 sequences were obtained for each accession. Phylogenetic trees were constructed by Maximum Likelihood method to choose two sequences with less difference, which were used for the further analysis. However, two sequences with less difference were in one clade for some accessions, eg. *P. bretschneideri* (Cili), *P. pyrifolia* (Manding xueli, Hepi li), *P. ussuriensis* (Xiaoxiangshui, Manyuanxiang), *P. Sinkiangensis* (Kuerlexiangli), *P. dimorphophylla* 5, *P. betulaeformia* 'QS3', *P. xerophila* 3, *P. pyrastrer* 1671, *P. regelii* 2513, *P. salicifolia* 2427, *P. syriaca* 908, *P. spinosa* 1516, *P. mamorensis* 1622, *P. communis* 'comice', more clones were needed to confirm if there was new genes, thus these species were not included in the following analysis and discussion except 'Kuerlexiangli'.

After preliminary selection, two copies were kept for each accession for further analysis. A total of 93 accessions in *Pyrus* were used for constructing phylogenetic trees by Maximum Likelihood. The results separated the Oriental pear (A) from Occidental pear (B), of which there were seven clades (A1-7, B1-7) for them, respectively (Figure 5). Accessions with same Indels were clustered in the same clade (Figure 5), such as accession copy in clade A1 had one T missing (Del 1(T), and so forth); Del 1 (A) were showed in clade A2 and A3 except for *P. pyrastrer* 989_2; There were Del 6 (GGAGCT) and Del 1 (A) in clade A4, and Del 1 (C) in clade A5. Del 9 (CGCATATCA) was in clade B1, Del 4 (GTGT) in B2, Del 15 (TCCTTGAGCCTTGGA) in B6, Ins 5 (TTTCA) in B7b, Del 13 (CACAAGAATCTTC) in B7b. Though both 'Yali' and *P. caucasica* 684 had Del 6 (GGAGCT) and Del 1 (A), they did not joined together with *P. ussuriensis* (A4) missing these two Indels, which might be caused by more SNPs difference in *P. ussuriensis*.

The phylogenetic trees constructed by NIA had many polytomies, for instance, *P. bretschneideri*, *P. pyrifolia* and *P. ussuriensis* were still mingled together in clade A1 and A3, while European species, West Asian species and North African species in clade B5 were still not separated. The results also showed coevolution of species, for instance, one copy of *P. ussuriensis* (A4), *P. calleryana* (A2), *P. pashia* (A5), *P. syriaca* (B2), *P. regelii* (B3) and North African species (B7b) were in the same clade. In addition, two copies from part of *P. ussuriensis* accessions and both of two *P. xerophila* accessions were clustered in clade A3 and A4, respectively, demonstrating *P. xerophila* had close relationships with *P. ussuriensis*.

In Oriental pear, (1) majority of two copies of Chinese White Pear and *P. Pyrifolia* were in clade A1 and A3, the rest were in clade A2 (CWP 'Huangjitui'_1, *P. pyrifolia* 'Dalainaitou'_2), A5 (*P. pyrifolia* 'Dalihuoba'_1), A7 (CWP 'Yali'_2); (2) two copies of *P. ussuriensis* were mainly in clade A1, A3/A4, but two copies of *P. ussuriensis* 'Manyuanxiang' were in clade A3 and A4, respectively; (3) both copies of *P. Sinkiangensis* were in clade A1; (4) all copies of *P. pashia* were in clade A5, except for *P. pashia* 'P10-3'_1 (A3) and *P. pashia* 'P23-4'_1 (A6); (5) both copies of *P. betulaeformia* were in clade A3; (6) all copies of *P. calleryana* were in clade A1 and A2 except for *P. calleryana*_1 (A7), *P. calleryana* 'DP8-1'_1 (A5) and *P. calleryana* '58'_2 (A3); (7) both copies of *P. xerophila* were in clade A3 and A4; (8) both copies of *P. hondoensis* were in clade A3 and A4; (9) 'Yali' (Del 6), *P. calleryana*_1 and *P. caucasica* 684 (Del 6) were in clade A7, which was at the bottom of the tree.

In Occidental pear, (1) clade B1 were composed of *P. regelii* 2587_1, *P. nivalis* 1590_1 and *P. elaeagnifolia* 1278_2 with the 9bp deletions (Del 9), which was at the bottom of the tree; (2) ten accessions with 4bp deletions (Del 4) were in clade B2, including European species, West Asia species, North African species; (3) *P. regelii* comprised of clade B3; (4) clade B4 and B5 included all accessions from Occidental pear, of which all copies were from West Asian species and North African species except for *P. nivalis* 725_2 in clade B4; (5) both copies of three *P. nivalis* accessions, *P. spinosa* 1615_2 and *P. salicifolia* 2797_1 with Del 15 and Del 2 were in clade B6; (6) North African species (*P. cossonii*, *P. mamorensis*) were in clade B7b with 5bp insertions (Ins 5) in copies; (7) the copies in clade B7c had 13bp deletions except for *P. spinosa* 1598_2 (Del 1).

3.4.2 The phylogenetic relationships of interspecies in Oriental pear

The phylogenetic relationships of Chinese White Pear, *P. pyrifolia* and *P. ussuriensis* were still not well solved (Figure 5). *P. pyrifolia*, some accessions from *P. ussuriensis* and Chinese White Pear, were mingled together (A1, A3), and accessions could not be well distinguished. However, some accessions of *P. ussuriensis* had close relationships with *P. xerophila* 2 and *P. xerophila* 4 (A3, A4). *P. ussuriensis* and *P. xerophila* distributed in the north of China, they had overlapped area in geography, which also suggested *P. ussuriensis* was different from *P. pyrifolia* and Chinese White Pear. *P. xerophila* 2 might be interspecific hybrid between Oriental pear and Occidental pear, of which the maternal parent was Oriental pear, and the male parent might be Occidental pear or at least had complex background referring to Occidental pear (Zheng et al. 2014). Considered of the results here, one of parents might be *P. ussuriensis*, therefore, more accessions from *P. xerophila* and other species of *Pyrus* were needed to validate further.

P. betulaeformia, *P. calleryana* and *P. pashia* were primitive species. The number of ventricles in *P. betulaeformia* and *P. calleryana* were 2~3, while that in *P. pashia* were 3~5, which probably suggested *P. betulaeformia* and *P. calleryana* were more primitive species. Three accessions of *P. betulaeformia* from Heinan Queshan (QS), Gansu Ningxian (NX) and Shandong Chengyang (CY) were clustered in different subclades of A3, having close genetic relationships with *P. pyrifolia*. However, they are in different clades (Group I, Group III, Group IV) in the NJ tree for *P. betulaeformia* populations based on *accD-psal* (Zong et al., 2014). The samples were widely chosen in this study and differences among intraspecies were more outstanding, so did *P. calleryana* and *P. pashia*. Majority of wild pea pear from Zhejiang province were in clade A2, which formed polytomies with other Oriental pears except from *P. pashia*. Most copies of wild *P. pashia* from Yunnan province were in clade A5, which connected Oriental pear and Occidental pear. Furthermore, *P. pashia* 'P10-3'_1 clustered with other Oriental pear in clade A3, while *P. pashia* 'P23-4'_1 and Occidental pear 'comice' were in clade A6, indicating wild *P. pashia* might be transitional species between Occident pears and Oriental pears, and more primitive speices might be from southwest mountain are by evolution (Rubtsov, 1944).

Though *P. dimorphophylla* and *P. calleryana* were similar in morphology, they were different species. Zheng et al. (2014) thought that the parents of *P. dimorphophylla* 5 (haplotype tH3aH2) and *P. dimorphophylla* 6 (tH1aH6) were distinct. Both copies of *P. dimorphophylla* 5 were in clade A3, and they were only closed with *P. calleryana* 58_2. One copy of *P. dimorphophylla* 6 had close relationship with *P. calleryana* 58_2, while the other one was with most of wild pea pear in clade A2, which suggested that *P. dimorphophylla* 5 and *P. dimorphophylla* 6 were probably from distinct parents, and they might have close genetic relationship with wild pea pear.

3.4.3 The phylogenetic relationships of interspecies in Occidental pear

In Occidental pear, two species of *P. communis* (*P. caucasica* and *P. pyraister*) contained one probable interspecific hybrid between Oriental pear and Occidental pear (*P. caucasica* 684, *P. pyraister* 989). Copies of *P. pyraister* 881 (B5, B7c) were in different clades, illustrating a complicated genetic background. There were rich Indels (Del 2, Del 4, Del 9, Del 11, Del 15, Ins 1(A)) in three accessions of European species *P. nivalis*. Two copies of *P. nivalis* 1590 were in clade B1 (Del 9) and B6 (Del 2 and Ins 1(A)), with close relationships with West Asian species except for *P. syriaca*. *P. nivalis* 256 and *P. nivalis* 1196 were thought to be hybrids between *P. amygdaliformis* and *P. nivalis*. Two copies of them were both in clade B2 (Del 4, Del 11) and B6 (Del 15), with close relationships with *P. syriaca* and *P. spinosa*, which was in accordance with previous results (Zheng et al., 2014).

The leaf characters were variable in West Asian species (*P. elaeagnifolia*, *P. salicifolia*, *P. regelii*, *P. spinosa*, *P. syriaca*). Most of them were named after leaf shape, and they had complex phylogenetic relationships and mingled with West Asian species and North African species in the phylogenetic trees. *P. elaeagnifolia* had complicated genetic background, and their copies were in clade B1, B2, B4, B5, B7, A2. *P. elaeagnifolia* 2817, a possible interspecific hybrid between Oriental species and Occidental species, had copies in clade A2 and B4. *P. elaeagnifolia* 1278 was closed with *P. regelii*, *P. nivalis* and *P. spinosa* and had copies in clade B1 and B7. *P. salicifolia* was rich in Indels (Del 2, Del 4, Del 13, Del 15), and copies were in B2, B4, B5, B6, B7, indicating an abundant background. Though the leaf shape of *P. salicifolia* 2427 was distinct from other accessions of *P. salicifolia*, two copies of it were clustered with copies from other accessions of *P. salicifolia* in clade B7. *P. regelii* was one of the most primitive species, and one copy of three accessions from *P. regelii* was in clade B2, which comprised of polytomies with other clades of Occidental pear. The phylogenetic position of *P. regelii* was unclear, which need more gene sequences to illustrate. *P. spinosa* was thought to be simple background, however, relationships were showed with *P. nivalis* and other West Asian species. One parent of *P. spinosa* 1598 was *P. betulifolia* possibly (Jiang et al., 2014), but both copies were in clade B7 without lineage of Oriental pear, more clones were needed to verify. One copy of three accessions from *P. syriaca* were in clade B2 (Del 4), the other one was in clade B2 (Del 11), B5 and B7 (Del 13), demonstrating intimate relationships with *P. salicifolia*.

It was reported that *P. cossonii* was differentiated from *P. mamorensis*, *P. gharbiana* in North African species, because of no flavonoid glycosides in *P. mamorensis*, or the phylogenetic results from *LFY2int2-N*. For these three North African species, the haplotypes were different (cH12, cH8, cH13 and cH1) based on *ndhC-trnV*, while *P. gharbiana* and *P. mamorensis* had the same haplotype of rH11 based on *trnR-atpA*. However, there was Ins 5 in all accessions of North African except *P. gharbiana* 834 and *P. mamorensis* 1622 based on NIA-i3, of which these copies were in clade B7. Two copies of two accessions of *P. gharbiana* were in clade B5 and B7, suggesting they had simple background. For *P. cossonii*, *P. cossonii* 753 and two accessions of *P. gharbiana* were in the same cluster, suggesting intimate genetic relationships of them. One copy of *P. cossonii* 829 had Del 13, with close relationships with *P. pyraister* and *P. salicifolia*. Copies of the rest two accessions from *P. cossonii* and two accessions from *P. mamorensis* were in clade B4 and B7, with close relationship. *P. mamorensis* 834 and *P. elaeagnifolia* were closely related. All of these results indicated intimate relationships of these three species.

3.4.4 The classification of hybrids between Oriental pear and Occidental pear and synonym

Two copies of *P. hondoensis* and Occidental pear *P. caucasica* 684, *P. pyraister* 989, *P. elaeagnifolia* 2817 were in clade A and B, respectively, indicating they were possible hybrids between Oriental pear and Occidental pear.

P. caucasica 684 was a hybrid originated from Ukraine, of which the maternal parent was probably *P. xerophila* of Oriental pear, and the male parent was probably *P. cossonii* and *P. caucasica* (Zheng et al., 2014). One copy of *P. caucasica* 684 was in clade A7 with Del 6 and Del 1 (A) which were same as *P. ussuriensis* and *P. xerophila*, while the other copy was in clade B5 with *P. cossonii*, *P. caucasica*. The maternal parent of *P. caucasica* 684 was proposed to be *P. betulifolia* (cH4rH18) or *P. xerophila* or *P. ussuriensis* according to the results from two chloroplast intergenic regions of *ndhC-trnV* and *trnR-atpA*. *P. pyraister* 989 was differed from the other two accessions of *P. pyraister* with the haplotype of tH15aH18 on the basis of *accD-psaI* and *trnL-F*. One copy of *P. pyraister* 989 was clustered with *P. pyrifolia*, *P. dimorphophylla* and *P. betulifolia*, the other copy was in clade B5 with other Occidental pear species which was differed from the other two accessions from *P. pyraister*. However, the parent of *P. pyraister* 989 was unknown, more accessions from other species were needed.

West Asian species *P. elaeagnifolia* 2817 had two copies in clade A2 and B4, respectively, with intimate relationships with wild pear and *P. salicifolia* or *P. caucasica*. However, It only had close relationships with *P. salicifolia*, and no evidence showed it was a hybrids between Oriental pear and Occidental pear. *P. hondoensis* was similar with *P. ussuriensis* in morphology. One copy in clade A3 was closely related with Chinese White Pear, *P. pyrifolia* and *P. ussuriensis*, the other one in clade B4 had intimate relationships with *P. salicifolia*, *P. elaeagnifolia*, *P. caucasica* and *P. mamorensis*. There was no report that *P. elaeagnifolia* 2817 and *P. hondoensis* were hybrids, more genes were needed.

'Kuerle Xiangli' was thought to be hybrid between Oriental pear and Occidental pear all the time. Though 10 clones were sequenced, no sequence was in clade with Occidental pear, probably only one copy was exist, or other copy could not obtained, more clones need to be sequenced.

P. elaeagnifolia 768, *P. regelii* 890 and *P. cossonii* 828 were in different clades, however, they were thought to be synonym (Jiang et al., 2014), which was not accordance with results of *ndhC-trnV* and *trnR-atpA*.

Conclusion

This paper supported genetic diversity of *Pyrus* in the view of molecular biology. The Neighbor-Net phylogenetic network indicated that the phylogenetic relationships were complicate based on *ndhC-trnV* and *trnR-atpA*, which the interspecies and intraspecies of Oriental pear and Occidental pear were intercrossed. Oriental pear and Occidental pear were separated in the tree of NIA-i3, *P. betulifolia*, *P. pashia* (except *P. pashia* 'P10-3'_1 and *P. pashia* 'P23-4'_1) were monophyly; several *P. ussuriensis* were closely related with *P. xerophila*. However, the phylogenetic relationships of Chinese White Pear, *P. pyrifolia* and

some *P. ussuriensis* could not be well solved; West Asian species and European species were mingled together, which might caused by few genes and a few of accessions of each species. Occidental pear *P. caucasica* 684, *P. pyraeaster* 989, *P. elaeagrifolia* 2817 might be hybrids between Oriental pear and Occidental pear. More genes and more accessions were needed to solve the phylogenetic relationships in *Pyrus* and to explore the possible parents.

Abbreviations

NIA: Nitrate Reductase; cH: ndhC-trnV haplotype; rH: trnR-atpA haplotype; Del: Deletion; Ins: Insertion; MP: Maximum parsimony; TBR: Tree-Bisection-Reconnection; ML: Maximum likelihood; GTR: general time reversible model; MCMC: Markov Chain Monte Carlo.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the findings of this work are available within the paper. The datasets analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y.T. designed the experiments. D.C. wrote the main manuscript text. S.J. and X.Y. did the DNA extraction. S.J., P.S. and D.C. performed the experiments of PCR and sequencing. D.C. and X.Z. did the phylogenetic analysis.

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Tables

Table 1 Accessions’ information in this study.

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

‘√’ stands for bands obtained by corresponding accession and primer.

Table 2 Primer sequences for three chloroplast regions and NIA.

Primer name	Forward5’-3’	Reverse5’-3’
<i>ndhC-trnV</i>	CTAATCGGGGCTAAACTCC	CCACATAATGAATCAGAGCACA
<i>trnR-atpA</i>	CGCTACCCGCTTTTACTTTA	CCAAGACATTTACCGACGAA
NIA-i3	AARTAYTGGTGYTGGTGYTTYTGCTC	GAACCARCARTTGTTTCATCATDCC

Table 3 Polymorphism of haplotypes and nucleotides for two chloroplast regions

Primer name	Number of Haplotypes (H)	Haplotype diversity (Hd)	Nucleotide diversity (Pi)	Average number of nucleotide differences (k)	Tajima’s test (D)	P value
<i>ndhC-trnV</i>	17	0.752	0.00325	1.54910	-1.88041	*P < 0.05
<i>trnR-atpA</i>	20	0.720	0.00923	3.590	-1.53301	P > 0.10

* stands for significant difference.

Table 4 The haplotype of *ndhC-trnV* and the number of haplotypes in each species of *Pyrus* L.

Accession's name	Sample code	<i>ndhC-trnV</i>	<i>trnR-atpA</i>	NIA-i3	Origin	Leaf source
Oriental species						
Chinese white pear						
'Fenhongxiao'	DF1	√	√	√	Hebei	CPGR
'Fengxianjitui '	DF2	√	√	√	Shanxi	ZZFI
'Jingchuan'	DF3	√	√	√	Gansu	CPGR
'Yinbai'	DF4	√	√	√	Hebei	CPGR
'Xuehua'	DF5	√	√	√	Hebei	CPGR
'Xiangchun'	DF6	√	√	√	Shanxi	CPGR
'Huangjitui'	DF7	√	√	√	Jiangsu	CPGR
'Eli'	DF8	√	√	√	Henan	CPGR
'Yali'	DF9	√	√	√	Hebei	TU
'Dadongguo'	DF10	√	√	√	Gansu	GPI
'Cili'	DF11	√	√	√	Shandong	CPGR
'Duanbajitui'	DF12				Sichuan	CPGR
'Haitangsu'	DF13	√			Jiangsu	CPGR
'Qingpicao'	DF14		√		Unknown	
<i>Ppyrifolia</i> Naikai						
'Shengxianshali'	DF15	√	√	√	Zhejiang	CPGR
'Rentouli'	DF16	√	√	√	Zhejiang	WSGR
'Dalihuoba'	DF17	√	√	√	Yunnan	WSGR
'Manding Xueli'	DF19	√	√	√	Fujian	CPGR
'Pugua'	DF20	√		√	Zhejiang	WSGR
'Nuodao'	DF21			√	Zhejiang	WSGR
'Huahong'	DF22	√		√	Zhejiang	WSGR
'Yandangxueli'	DF23			√	Zhejiang	WSGR
'Dahuangcha'	DF24			√	Zhejiang	WSGR
'Jiuzhong'	DF25			√	Zhejiang	WSGR
'Ishiharawase'	DF18	√	√	√	Japan	TU
'Nijisseiki'	DF26	√	√	√	Chiba, Japan	TU
'Hakataao'	DF27	√	√	√	Fukuoka Pref., Japan	TU
'Imamuraaki'	DF28	√	√	√	Takashi Pref., Japan	TU
'Hakuteiryu'	DF29	√	√	√	Niigata Pref.	TU
'Chojuro'	DF30			√	Kanagawa	TU
'Yiwulizi'	DF31			√	Zhejiang	CPGR
'Baozhuli'	DF55	√	√	√	Yunnan	WSGR
'Damali'	DF56	√	√		Sichuan	WSGR
'Hongshaobang'	DF57	√	√		Sichuan	WSGR
'Muguali'	DF58	√	√	√	Guizhou	wild
'Wuli'	DF59	√			Guizhou	wild
'Hepili'	DF60	√	√	√	Guangxi	wild
'Shuinanli'	DF61	√	√	√	Guangxi	wild
'Dalinaitou'	DF62	√		√	Yunnan	WSGR

<i>Pussuriensis</i> Masxim						
‘Jianbali’	DF32	√		√	Liaoning	TU
‘Manyuanxiang’	DF33	√		√	Liaoning	CPGR
‘Balixiangli’	DF34	√		√	Liaoning	CPGR
‘Hongbalixiang’	DF35	√	√	√	Liaoning	CPGR
‘Huagai’	DF36	√	√	√	Liaoning	CPGR
‘Tianqiuzi’	DF37	√	√	√	Liaoning	CPGR
‘Saozhoumiaozhi’	DF38	√	√	√	Liaoning	CPGR
‘Xiehuatian’	DF39	√	√	√	Liaoning	CPGR
‘Ruanerli’	DF40	√		√	Gansu	GPI
‘Xiaoxiangshui’	DF41	√		√	Liaoning	CPGR
‘Qingmian’	DF42			√	Hebei	CPGR
‘Yaguang’	DF43	√	√	√	Liaoning	CPGR
‘Nanguoli’	DF44	√	√		Liaoning	TU
‘Daxiangshui’	DF45	√		√	Liaoning	CPGR
‘Jinxishanli’				√	Liaoning	CPGR
<i>Psinkiangensis</i> Yu						
‘Korlaxiangli’	DF46		√	√	Xinjiang	CPGR
<i>Pdimorphophylla</i> Makino						
<i>Pdimorphophylla</i> 6	DF47	√	√	√	Japan	TU
<i>Pdimorphophylla</i> 5	DF48	√		√	Japan	TU
<i>Pxerophila</i> Yu						
<i>Pxerophila</i> 1	DF49	√		√	Gansu	GPI
<i>Pxerophila</i> 2	DF50			√	Gansu	GPI
<i>Pxerophila</i> 3	DF51			√	Gansu	GPI
<i>Pxerophila</i> 4	DF52	√		√		
<i>Phondoensis</i> Yu	DF53			√	Central Japan	TU
<i>Phopeiensis</i> Yu						
<i>Phopeiensis</i>	DF54	√	√	√	Hebei	Hebei
<i>Pbetulaefolia</i> Bge.						
BT1	DL1	√	√	√	Baota, Shanxi	wild
NQ11	DL2	√		√	Neiqiu, Hebei	wild
BT10	DL3			√	Baota, Shanxi	wild
BT11	DL4	√		√	Baota, Shanxi	wild
BT13	DL5			√	Baota, Shanxi	wild
FX1	DL6			√	Fuxian, Shanxi	wild
BT21	DL7			√	Baota, Shanxi	wild

BT8	DL8			√	Baota, Shanxi	wild
BX6	DL9	√		√	Binxian, Shanxi	wild
CL1	DL10	√		√	Changli, Hebei	wild
CL3	DL11	√	√	√	Changli, Hebei	wild
CY1	DL12	√		√	Chengyang, Shandong	wild
FX11	DL13	√		√	Fuxian, Shanxi	wild
HC12	DL14	√		√	Huachi, Gansu	wild
SO13	DL15	√		√	Songxian, Henan	wild
NX18	DL16			√	Ningxian, Gansu	wild
QS1	DL17		√	√	Queshan, Henan	wild
QS4	DL18			√	Queshan, Henan	wild
QS5	DL19	√		√	Queshan, Henan	wild
QS3	DL20	√		√	Queshan, Henan	wild
ZN18	DL21	√		√	Zhengning, Gansu	wild
'Lushiduli'		√	√		Zhejiang University	

***P.calleryana* Dcne**

<i>P. calleryana</i> 6	DL22	√	√	√	Zhejiang	wild
<i>P. calleryana</i> 16	DL23	√		√	Zhejiang	wild
<i>P. calleryana</i> 32	DL24			√	Zhejiang	wild
<i>P. calleryana</i> 52	DL25	√		√	Zhejiang	wild
<i>P. calleryana</i> 53	DL26			√	Zhejiang	wild
<i>P. calleryana</i> 58	DL27			√	Zhejiang	wild
<i>P. calleryana</i> 89	DL28			√	Zhejiang	wild
<i>P. calleryana</i> DP8-1	DL29	√	√	√	Zhejiang	wild
<i>P. calleryana</i> DP8-3	DL30	√	√	√	Zhejiang	wild
<i>P. calleryana</i> DP7-7	DL44	√	√	√	Zhejiang	wild
<i>P. calleryana</i>				√	Zhejiang	wild

***P.pashia* D.Don**

<i>P. pashia</i> P1-1	DL31	√	√	√	Yunnan	wild
<i>P. pashia</i> P1-2	DL32	√	√	√	Yunnan	wild
<i>P. pashia</i> P1-3	DL33	√		√	Yunnan	wild
<i>P. pashia</i> P1-7	DL34			√	Yunnan	wild
<i>P. pashia</i> P1-9	DL35	√	√	√	Yunnan	wild
<i>P. pashia</i> P5-11	DL36	√	√	√	Yunnan	wild
<i>P. pashia</i> P7-6	DL37	√		√	Yunnan	wild
<i>P. pashia</i> P10-3	DL38	√		√	Yunnan	wild
<i>P. pashia</i> P12-7	DL39			√	Yunnan	wild
<i>P. pashia</i> P15-5	DL40	√		√	Yunnan	wild
<i>P. pashia</i> P20-3	DL41	√	√	√	Yunnan	wild
<i>P. pashia</i> P23-4	DL42	√		√	Yunnan	wild
<i>P. pashia</i> P23-5	DL43	√		√	Yunnan	wild

Unknown species						
'Bali1'		✓	✓		Pakistan	wild
'Bali2'		✓	✓		Pakistan	wild
'Bali3'		✓	✓		Pakistan	wild
'Bali4'		✓	✓		Pakistan	wild
'BaR10'					Pakistan	wild
Occidental species						
European species						
<i>Pcommunis</i> L.						
<i>Pcaucasica</i> 681	XF62			✓	Unkown	NCGR, PI=541563
<i>Pcaucasica</i> 684	XF1	✓	✓	✓	Ukraine	NCGR, PI=322285
<i>Pcaucasica</i> 687	XF2	✓	✓	✓	Russia	NCGR, PI=337437
<i>Pcaucasica</i> 694	XF3	✓		✓	Russia	NCGR, PI=440631
<i>Pcaucasica</i> 2816	XF7	✓		✓	Armenia	NCGR, PI=638008
<i>Pcaucasica</i> 2866	XF55			✓	Tavush Marz, Armenia	NCGR,Q=43814
<i>Ppyraster</i> 881	XF52	✓	✓	✓	Uncertain	NCGR, PI=541567
<i>Ppyraster</i> 989	XF4	✓		✓	Yugoslavia	NCGR, PI=349026
<i>Ppyraster</i> 1288	XF5	✓	✓	✓	Iran	NCGR, PI=132094
<i>Ppyraster</i> 1671	XF6	✓		✓	Romania	NCGR, PI=506380
<i>Pcommunis</i> 'casacade'		✓	✓		unknown	
<i>Pcommunis</i> 'comice'		✓	✓	✓	unknown	
<i>Pnivalis</i> Jacq						
<i>Pnivalis</i> 256	XF8	✓	✓	✓	Netherland	NCGR, PI=312144
<i>Pnivalis</i> 725	XF9	✓	✓	✓	Europe	NCGR, PI=541859
<i>Pnivalis</i> 1196	XF10	✓		✓	Netherlands	NCGR, PI=541864
<i>Pnivalis</i> 1590	XF11	✓	✓	✓	Yugoslavia	NCGR, PI=541867
<i>Pnivalis</i> 1714	XF12				Uzbekistan	
<i>Pnivalis</i> 256.002 (2)	XF60	✓	✓	✓	Netherland	NCGR, PI=312144
<i>Pnivalis</i> 725 (2)	XF63			✓	Europe	NCGR, PI=541859
<i>Pnivalis</i> 862	XF51	✓	✓	✓	unknown	NCGR, PI=541860
<i>Pcordata</i> (Desv.) Schneider						
<i>Pcordata</i> 739	XF13	✓	✓	✓	Turkey	NCGR, PI=541571
<i>Pcordata</i> 745	XF14	✓		✓	France	NCGR, PI=617506
<i>Pcordata</i> 750	XF15	✓	✓	✓	France	NCGR,PI=541580
<i>Pcordata</i> 1588	XF16	✓		✓	Turkey	NCGR,PI=541590
West Asian species						
<i>Pelaeagrifolia</i> Pall.						
<i>Pelaeagrifolia</i> 768	XF17	✓		✓	Turkey	NCGR, PI=541613
<i>Pelaeagrifolia</i> 904	XF18	✓		✓	Wild Hungary	NCGR, PI=312154
<i>Pelaeagrifolia</i> 1278	XF66	✓	✓	✓	Turkey	NCGR, PI=541616

<i>Pelaeagrifolia</i> 1482	XF19	✓		✓	Crimea Ukraine	NCGR, PI=293846
<i>Pelaeagrifolia</i> 1490	XF20	✓	✓	✓	Turkey glabrous	NCGR, PI=617525
<i>Pelaeagrifolia</i> 1711	XF21			✓	Uzbeksitan hybrid	NCGR, PI=541630
<i>Pelaeagrifolia</i> 2817	XF22			✓	Armenia	NCGR, PI=638009
<i>Pelaeagrifolia</i> 768 (2)	XF64	✓	✓	✓	Turkey	NCGR, PI=541613
<i>Pelaeagrifolia</i> 904 (2)	XF65	✓	✓	✓	Hybrid, Hungary	NCGR, PI=312154
<i>Pelaeagrifolia</i> 1482 (2)	XF67			✓	Crimea	NCGR, PI=293846
<i>Pelaeagrifolia</i> 1482.001(2)	XF53	✓	✓	✓	Crimea	NCGR, PI=293846

***Pspinosa* (*P. amygdaliformis* Vill.)**

<i>P.spinosa</i> 634	XF58	✓	✓	✓	Turkey	NCGR, PI=540929
<i>P.spinosa</i> 636	XF59			✓	Macedonia	NCGR, PI=349021
<i>Pspinosa</i> 639	XF61	✓	✓	✓	Greece	NCGR, PI=540922
<i>Pspinosa</i> 1598	XF23			✓	Sardinia, Italy	NCGR, PI=540934
<i>P.spinosa</i> 1608	XF24	✓	✓	✓	Macedonia, Greece	NCGR, PI=540937
<i>Pspinosa</i> 1610	XF25	✓		✓	Turkey	NCGR, PI=540939
<i>Pspinosa</i> 1615	XF26			✓	Macedonia	NCGR, PI=349023

***Pregelii* Rehd.**

<i>Pregelii</i> 890	XF27	✓	✓	✓	Russia	NCGR, PI=541945
<i>Pregelii</i> 2513	XF28			✓	Kazakhstan	NCGR, PI=617589
<i>Pregelii</i> 2587	XF29			✓	Kazakhstan	NCGR, PI=641279

***Psalicifolia* Pall.**

<i>Psalicifolia</i> 869	XF30	✓	✓	✓	Uncertain	NCGR, PI=541950
<i>Psalicifolia</i> 2382	XF31	✓	✓	✓	Hybrid	NCGR, PI=617559
<i>Psalicifolia</i> 2427	XF69	✓	✓	✓	Russia	NCGR, Q=29557
<i>Psalicifolia</i> 2720	XF32	✓	✓	✓	Armenia	NCGR, PI=638000
<i>Psalicifolia</i> 2797	XF33	✓	✓	✓	Georgia	NCGR, PI=657921
<i>Psalicifolia</i> 2849	XF34		✓	✓	Russia	CPYR=2720
<i>Psalicifolia</i> 2883	XF56			✓	Georgia	NCGR, PI=657924

***Psyriaca* Boiss**

<i>Psyriaca</i> 908	XF35	✓		✓	Israel	NCGR, PI=541975
<i>Psyriaca</i> 914	XF36	✓	✓	✓	Israel	NCGR, PI=541977
<i>Psyriaca</i> 920	XF37	✓	✓	✓	Armenia	NCGR, PI=312149
<i>Psyriaca</i> 2716	XF38	✓	✓	✓	Israel	NCGR, PI=641281

North African species

***Pcossonii* Rehd.**

<i>Pcossonii</i> 753	XF39			✓	Morocco	NCGR, PI=316552
<i>Pcossonii</i> 828	XF40	✓		✓	Unkown	NCGR, PI=541592
<i>Pcossonii</i> 829	XF41	✓		✓	Unkown	NCGR, PI=541593
<i>Pcossonii</i> 830	XF42			✓	Unkown	NCGR, PI=541592

<i>P.gharbiana</i> Trab						
<i>P.gharbiana</i> 789	XF43	✓	✓	✓	Morocco	NCGR, PI=541663
<i>P.gharbiana</i> 790	XF44	✓	✓	✓	Morocco	NCGR, PI=541664
<i>P.gharbiana</i> 794	XF45	✓		✓	Morocco	NCGR, PI=541666
<i>P.gharbiana</i> 787	XF57			✓		NCGR, PI=541662
<i>P.gharbiana</i> 1621	XF54	✓	✓	✓		NCGR, PI=541669
<i>P.mamorensis</i> Trab						
<i>P.mamorensis</i> 834	XF46	✓	✓	✓	Morocco	NCGR, PI=541843
<i>P.mamorensis</i> 835	XF47			✓	Morocco	NCGR, PI=541844
<i>P.mamorensis</i> 837	XF48	✓	✓	✓	Morocco	NCGR, PI=541846
<i>P.mamorensis</i> 841	XF50	✓	✓	✓	Morocco	NCGR, PI=541847
<i>P.mamorensis</i> 1622	XF49		✓	✓	Unknown	NCGR, PI=541855
Outgroup species						
<i>Malus.sieboldii</i> (Regel.) Rehder		✓			Yunnan	HRIYN
<i>Malus.domestica</i> Ralls'					Yunnan	HRIYN
<i>Malus pumila</i>			✓		Unknown	Unknown

Haplotype	Hap1	Hap2	Hap3	Hap4	Hap5	Hap6	Hap7	Hap8	Hap9	Hap10	Hap11	Hap12	Hap13	Hap14	Hap15
Chinese White Pear		4		9											
<i>P. pyrifolia</i>		9		9				2							
<i>P. ussuriensis</i>		10		3											
<i>P. sinkiangensis</i>			1												
<i>P. betulaeifolia</i>				14											
<i>P. calleryana</i>				5				1							
<i>P. dimorphophylla</i>		1		1											
<i>P. pashia</i>				10		1	1								
<i>P. xerophila</i>				2											
<i>P. hopeiensis</i>				1											
Unkown (Pakistan)					1			1							1
<i>P. caucasica</i>				1				3							
<i>P. pyraister</i>								3						1	
<i>P. cordata</i>								2							2
<i>P. nivalis</i>								5	1						
<i>P. elaeagrifolia</i>								5	4						
<i>P. spinosa</i>								4							
<i>P. regelii</i>								1							
<i>P. salicifolia</i>								5			1				
<i>P. syriaca</i>								3						1	
<i>P. cossonii</i>												2			
<i>P. gharbiana</i>								4							
<i>P. mamorensis</i>	2												1		
<i>P. communis</i>										1					
<i>Malus</i>	2														
Total	4	24	1	55	1	1	1	39	5	1	1	2	1	2	3

Table 5 The haplotype of *trnR-atpA* and the number of haplotypes in each species of *Pyrus* L.

Haplotype	Hap1	Hap2	Hap3	Hap4	Hap5	Hap6	Hap7	Hap8	Hap9	Hap10	Hap11	Hap12	Hap13	Hap14	Hap15
Chinese White Pear			12												
<i>P. pyrifolia</i>			14												
<i>P. ussuriensis</i>			7												
<i>P. sinkiangensis</i>			1												
<i>P. betulaefolia</i>			3												
<i>P. calleryana</i>			4												
<i>P. dimorphophylla</i>					1										
<i>P. pashia</i>			4					1							
<i>P. hopeiensis</i>			1												
Unkown (Pakistan)	3					1									
<i>P. communis</i>	1										1				
<i>P. caucasica</i>															1
<i>P. pyraister</i>	1						1								
<i>P. cordata</i>										1		1			
<i>P. nivalis</i>	3										1				
<i>P. elaeagrifolia</i>	1								1		2				
<i>P. spinosa</i>	1						1								
<i>P. regelii</i>															
<i>P. salicifolia</i>	4														2
<i>P. syriaca</i>											3				
<i>P. gharbiana</i>											3				
<i>P. mamorensis</i>											2		1	1	
<i>Malus</i>	1														
Total	1	14	46	1	1	1	2	1	1	1	12	1	1	1	3

Table 6 DNA alignment and tree statistics of the total datasets for the three chloroplast region and NIA-i3.

Region	Aligned character length	Number of sequences	No. of variable characters	No. of parsimony informative characters	Number of indel coded	Best Model	BIC Bayesian Information Criterion
<i>ndhC-trnV</i> CV	1066	143	179	948.8%	121	F81+G	7382.8729
<i>trnR-atpA</i> RA	1213	93	161	363.0%	120	HKY	5824.6525
NIA-i3	2118	277	628	43520.5%	108	TPM2uf+G	23737.6495

Figures

[illegible]

a) Neighbor-Net split graph of whole *Pyrus* based on trnR-atpA haplotypes. Different color stands for different splits, accession names were marked for all haplotypes except for three main haplotypes (cH2, cH4, cH8). b) Neighbor-Net split graph of trnR-atpA haplotypes based on NJ. The number in parentheses was the accession number for each haplotype.

Figure 5

Image not available with this version