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Original Article

AFLP Molecular Identification and Genetic Relationship of Chinese and Japanese Pear Cultivars Grown in Middle European Conditions

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Abstract

In this study, 30 genotypes of genus *Pyrus* (five European cultivars, 16 Asian cultivars, three rootstocks, four interspecific hybrids, one landrace cultivar from Czech Republic ('Krvavka'), *Pyrus betulaefolia* Bunge. and one intergeneric hybrid (*Cydomalus*)) were tested using AFLP markers. Twelve primer combinations generated a number of 1251 fragments of which 1064 were polymorphic with an average polymorphism of 85.3%. The dendrogram, created by using the UPGMA method, revealed a distinct genetic relationship between European and Asian pear groups. The intergeneric hybrid *Cydomalus* was separated in the cluster tree from both groups. The level of similarity coefficient between European and Asian pears was 0.75. Despite the fact that *Pyrus betulaefolia* Bunge was clustered in the European pear group, the average similarity coefficient between the European pear group and *Pyrus betulaefolia* Bunge (0.7704) was comparable to the similarity coefficient between the Asian pear group and *Pyrus betulaefolia* Bunge (0.768). Thus, the botanic species *Pyrus betulaefolia* Bunge can likely be considered as an intermediate genotype between European and Asian pears. The cultivar 'Talgarskaja Krasavica' (chance seedling of 'Forest Beauty'), which pomologically belongs to the European pear group was clustered together with the interspecific hybrid 'Wu Jiu Xinag' ('Ya Li' × 'Bartlett') which on the other hand belongs to the Asian pear group. Thus, due to its position in the dendrogram the cultivar 'Talgarskaja Krasavica' could be considered as an interspecific hybrid.

Keywords: AFLP, Asian pear, European pear, genetic relationship, *Pyrus*

Introduction

Pears are the third most important temperate fruit species after apples and grapes (Wu *et al.*, 2013). They belong to the family *Rosaceae*, subfamily *Pomoideae*, genus *Pyrus* ($2n$ = 34 chromosomes) (Jackson, 2003). *Pyrus* is a polymorphous genus from the northern hemisphere (Bailey, 1917) which includes 22 primary species, at least six naturally interspecific hybrids, and at least three artificial hybrids (Bell *et al.*, 1996). The genus *Pyrus* originates in Tertiary period (65-55 million years ago) in the foothills of the Tian Shan mountain massif in province Xinjang in western China. Progenitor pear genotypes can be found in the Chinese gene center, the central Asiatic gene center and the Near Eastern gene center (Vavilov, 1951).

Due to geographic and climatic factors, two groups were created: the Oriental (Asian) pear group and the Occidental (European) pear group (Bailey, 1917). The Oriental pear group is divided into five subgroups: Ussurian pears, Chinese white pears, Xinjiang pears, Chinese sand pears and Japanese pears. Cultivars of Ussurian pears undoubtedly originated from *Pyrus ussuriensis* Max, which naturally grows in northeastern China and the northern part of Heibei and Shanxi provinces (Shen, 1980). The group of Chinese white pears arose from *Pyrus* x *bretschneideri* Rehd. (*P. ussurinesis* × *P. betulifolia*). Japanese pears derived from wild *Pyrus pyrifolia* (Burm.) Nak., which originates in Middle and Southern Japan (Teng, 2004). Like Japanese pears, Chinese sand pears also arose from *Pyrus pyrifolia* (Burm.) Nak. (Shen, 1980). Japanese pears arose from progenitor genotypes which were introduced from ancient China via sea trade connecting Kochi Prefecture of Japan and Zhejing Province of China (Teng *et al.*, 2001, 2002; Shen *et al.*, 2006; Bao *et al.*, 2007, 2008). Xinjiang pears grown in Xinjiang Uygur Autonomous Region originate from *Pyrus* x *sinkiangensis* (*P. communis* L. × *P.* x *bretschneideri* Rehd.) (Teng *et al.*, 2001).

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The origins of pear growing in the Czech Republic have not been conclusively substantiated yet. One theory states that our ancestors gained knowledge of pear cultivation from neighboring Germanic countries or that they had direct contact with the Romans (Koch, 1967). The second theory says that pear trees were brought to our territory by Slavs during migrations between the 4th to 7th centuries. In the second half of the 19th century the most significant pear orchards were established in the Lower Elbe region. These orchards became the production and export centers for the Czech Republic and the famous Czech pears were crossing borders (Koch, 1967). In 1955 the annual harvest was 35,582 tons of pears (Koch, 1967); in 2010 the annual harvest of pears was only 16,157 tons, however in 2012 new orchards of total area of 326 ha were established in the Czech Republic (FAO, 2016).

In the last century numerous methods have been used for distinguishing the relatedness and origins of pear cultivars. Kikuchi (1946) and Yu (1979) described the taxonomy of Chinese and Japanese pears using morphological and pomological characters. Lin and Shen (1983) divided pear cultivars using peroxidase isozymic pattern and Zou *et al*. (1986) divided pear cultivars based on pollen ultrastructure. After the development of the PCR method a lot of molecular methods were developed, which are used to determine the genetic diversity, e.g. RAPD (Williams *et al.*, 1990; Welsh and McClelland M, 1990), SSR (Tautz *et al.*, 1986; Powell *et al.*, 1996) and AFLP (Vos *et al.*, 1995). For example, 20 RAPD markers were used for division of 72 Asian and European pears into 5 genetic groups (Teng *et al.,* 2001), while by use of 6 SSR markers another set of 98 *Pyrus* genotypes were divided into 10 groups (Bao *et al.,* 2007) and finally 6 AFLP markers used for division of 100 *Pyrus* genotypes created 15 groups (Bao *et al.,* 2008). Based on these methods, new relationships between cultivars and wild species were discovered. In spite of these advancements and findings, the taxonomy of genus *Pyrus* is still unstable (BAO *et al*., 2008).

Amplified Fragment Length Polymorphism (AFLP) is a suitable method for molecular identification in many studies concerning plants, animals, fungi, bacteria, and many other subjects in genetics, ecology, and evolution and was introduced in 1995 (Vos *et al.*, 1995). AFLP technology is a powerful DNA fingerprinting technique, which combines DNA restriction and PCR amplification (Cervera *et al.*, 1996). The main benefit of this method is its high polymorphism in plastid DNA unlike the SSR (Simple Sequence Repeats) method, which has really low polymorphism in the plastid DNA region (Bensch and Akesson, 2005).

The aim of this study was to assess the genetic relationship between cultivars of European pear and Asian pear groups grown in the Czech Republic. Several of the Asian pear group cultivars have had their names modified to Czech language in the past, consequently the original names are unknown along with their family genealogy. Determination of taxonomic relationships between pear cultivars, particularly Asian pear group cultivars grown in Czech Republic, can be useful to deduce their likely origin and to help in selection of progenitors for breeding programs.

Materials and Methods

Plant material

In the current study, 30 pear genotypes were evaluated including five European pear group cultivars, one landrace 'Krvavka' (from White Carpathians), 16 Asian pear group cultivars, three rootstocks, four interspecific hybrids, *Pyrus betulaefolia* Bunge, and the intergeneric hybrid *Cydomalus* (Table 1).

DNA isolation

Genomic DNA was isolated from young leaves. The DNA was isolated from 100 mg of crushed leaves using DNeasy Plant Mini Kit by Qiagen. The quality of isolated DNA was assessed by electrophoresis in 1% agarose gel and the concentration was measured by Modulus™ Single Tube Fluorometer 9200 – 000 (TURNER BIOSYSTEMS, USA).

AFLP analysis

For the restriction phase, 200 ng of genomic DNA was used. DNA was digested at 37 °C for 12 hours by a mixture of the restriction enzyme EcoRI (10 U) and MseI (2 U). After the restriction phase, DNA fragments were ligated to the EcoRI adaptor (5 pM) and the MseI adaptor (50 pM) in a mixture with 1 × NEB buffer 2, ATP (100 mM) and T4 DNA ligase (100 U) at 16 °C for 12 hours. Digest-ligated DNA fragments (primary templates) were diluted 10x from which 10 µl of was used in the pre-amplification phase. Total volume of PCR reaction was 50 µl containing: 1 × PCR buffer, dNTPs (25 mM), primer EcoRI – preamp (100 ng), primer MseI – preamp (100 ng), Taq polymerase (1.25 U) and HPLC water. The program consisted of 20 cycles at 94 °C for 45 seconds followed by 52 °C for 45 seconds and 72 °C for 60 seconds. The product of pre-amplification phase, the secondary template, was diluted 10×. For the last, selective amplification phase, twelve primer combinations were used (Table 2). Primer combinations consisted of four Mse I primers and three Eco RI primers which were labelled by fluorescent NED, FAM, and JOE dyes. Four sets of three variants were formed using one Mse I primer and three Eco RI primers.

The total volume of the selective amplification reaction was 15 µl consisting of 5 µl of secondary template, 1× PCR buffer, dNTPs (25 mM), primers Eco RI (5 pmol) and Mse I (15 pmol), Taq polymerase (2 U), and HPLC water. A touch-down PCR program was used consisting of 10 cycles at 94 °C for 30 seconds followed by 65 °C to 56 °C (decreasing by 0.7 °C in each consecutive cycle) for 30 seconds and 72 °C for 60 seconds, and then 24 cycles at 94 °C for 30 seconds followed by 56 °C for 30 seconds and 72 °C for 60 seconds.

PCR products from each set were mixed (6 µl NED + 4 µl FAM + 4 µl JOE). Two microliters of the sample mixture were mixed with 12 µl formamide and 0.5 µl GS ROX 500 size standard and were heat-denatured at 95 °C for five minutes and cooled down using ice. Samples were afterwards measured by genetic analyser ABI PRISM 310® (Applied Biosystems).

Data analysis

Data were analysed using the GeneScan Analysis® (Applied Biosystems) program. Samples were analysed for presence and absence of fragments. The results were written in a table as follows: 1 = presence of a fragment, 0 = absence of a fragment.

Table 1. *Pyrus* species included in the current study

Name	Species	Lineage	Origin
Cydomalus	Intergeneric hybrid	*Malus domestica* × *Cydonia oblonga*	Rusia
'Hosui'	*P. pyrifolia*	Ri - 14 ('Kikusui' × 'Yakumo') × 'Yakumo'	Japan 1954
'Chojuro'	*P. pyrifolia*	Chance seedling	Japan 1889
'Jinhua'	*P.* × *bretschneideri*	Unknown	Unknown
'Ju Li'	Unknown	Unknown	Unknown
'Kirgizkaja Zimnaja'	Unknown	Unknown	Unknown
'Kumt Ghant Chu'	Unknown	Unknown	Unknown
'Nijisseiki'	*P. pyrifolia*	Chance seedling	Japan 1898
'Ping Guo Li'	*P. ussuriensis*	Old selection from Beijing Municipality	China
'Pung Su'	Unknown	Unknown	Unknown
'Shin Li'	*P. pyrifolia*	'Kikusui' × 'Tsu Li'	California 1988
'Shinko'	*P. pyrifolia*	Chance seedling 'Nijisseiki'	Japan 1941
'Kumoi'	*P. pyrifolia*	'Ishii Wase' × 'Yakumo'	Japan 1955
'Shinseiki'	*P. pyrifolia*	'Nijisseiki' × 'Chojuro'	Japan 1945
'Shon Shu'	*P.x bretschneideri*	Unknown	China
'Talgarskaja Krasavica'	Unknown	Chance seedling Forest Beauty	Kazakhstan
'Wu Jiu Xiang'	Interspecific hybrid	'Ya Li' × 'Bartlett'	Unknown
'Xue Hua Li'	*P. x bretschneideri*	Unknown	Unknown
'Ya Li'	*P. x bretschneideri*	Old special, unknown	China
'Zao Su Li'	*P. x bretschneideri*	'Ping Guo Li' × 'Shenbuzhi'	China 1977
'Hood'	Interspecific hybrid	Oriental × Occidental hybrid	Florida
'Kieffer'	Interspecific hybrid	*P. pyrifolia* × 'Bartlett'	Philadelphia 1863
'Rafzas' syn. 'Benita'	Interspecific hybrid	*P. pyrifolia* × 'General Leclerc'	Switzerland
'Krvavka'	*P. communis*	Landrace special	White Carpathians
'Fox 11'	*P. communis* (rootstock)	Chance seedling from 'Volpina'	Italy
'Clapp's favourite'	*P. communis*	'Flemish Beauty' × 'Bartlett'	Massachusetts 1860 (USA)
'Conference'	*P. communis*	Seedling of 'Leon Leclerc de Laval'	England 1885
'Bartlett'	*P. communis*	Chance seedling	England 1770
'Pear seeding'	*P. communis* (rootstock)	Selection from old cultivars of pears	Czech Republic
'Pyrodwarf'	*P. communis* (rootstock)	'Old Home' × 'Bonne Luise d' Avraches'	Germany 1980
Pyrus betulaefolia	*P. betulifolia* (rootstock)	Botanical species	China

Table 2. The polymorphic characterization of AFLP primers in *Pyrus*

Primer combination Eco/Mse	Number of evaluated fragments	Polymorphic fragments	Percentage of polymorphic fragments (%)
E-ACT/M-TCGC	101	92	91.1
E-AGG/M-TCGC	108	99	91.7
E-AGC/M-TCGC	103	98	95.1
E-ACT/M-TCAA	110	99	90.0
E-AGG/M-TCAA	99	88	88.9
E-AGC/M-TCAA	111	99	89.2
E-ACT/M-GCAT	105	95	90.5
E-AGG/M-GCAT	95	84	88.4
E-AGC/M-GCAT	115	96	83.5
E-ACT/M-TACC	95	81	85.3
E-AGG/M-TACC	100	85	85.0
E-AGC/M-TACC	109	104	95.4
Total	1251	1120	
Average	104.3	93.3	89.5

Results had a form of a binary matrix. Similarities of obtained spectra between any two variants were evaluated by the Nei and Li/Dice similarity index method by FreeTree program (Hampl *et al.*, 2001). Using the cluster analysis method UPGMA (Bootstrap value 100) a dendrogram was constructed, which displays the degree of genetic similarity. Program TreeView X was used to achieve a better graphic representation.

Results

AFLP polymorphism

Regarding the AFLP polymorphism, from a total of 1 251 fragments (ranging from 90 to 500 bp) amplified with twelve primer combinations, a large amount of 1 120 (89.5%) fragments were polymorphic. The ability of individual primer pair to detect polymorphisms in the analysed genotypes is shown in Table 2. The highest polymorphism was found in primer combinations E-AGC/M-TCGC (98 polymorphic fragments, 95.1%) and E-AGC/M-TACC (104 polymorphic fragments, 95.4% respectively). In contrast, primer combinations E-AGC/M-GCAT had the lowest polymorphism (96 polymorphic fragments, 83.5%).

The cluster analysis

The genetic distance dendrogram separated European pears and Asian pears, showing 0.75 similarity index between these two groups. The dendrogram was divided into three main clusters. The first cluster split into three groups: groups I. and II. - Asian pears and group III. - interspecific hybrids. The second cluster was split into four groups: group IV. - *Pyrus betulaefolia* Bunge, group V. - interspecific hybrids, groups VI. and VII. - European pears. The level of similarity coefficient between Asian pears was 0.81 and 0.79 between European pears. The third, distinctly separated cluster was comprised of *Cydomalus,* an intergeneric hybrid (Fig. 1).

Group I. consisted of Japanese pears. Group II. consisted of two Japanese pears 'Kumoi' and 'Hosui', five Chinese white pears 'Xue Hua Li', 'Jinhua', 'Shon Shu', 'Ya Li', 'Zao Su Li', one Ussurien pear 'Ping gou Li', one Chinese sand pear 'Shin Li', and three pears with unknown origin 'Ju Li', 'Kumt Ghant Chu', and 'Pung Su'. The III. group consisted of 'Talgarskaja Krasavica' and 'Wu Jiu Xiang'. *Pyrus betulaefolia* Bunge was clustered in group IV. Three interspecific hybrids 'Hood', 'Kieffer' and 'Rafzas' created group V. Occidental pears clustered in Groups VI. and VII.

The highest similarity coefficient (0.8998) was recorded between Japanese pears 'Nijissejki' and 'Shinko'. The second highest similarity coefficient (0.89571) was found between Japanese pears 'Nijissejki' and 'Shinseiki'. The lowest similarity coefficients (0.62233 and 0.62763) were found between *Cydomalus*/'Talgarskaja Krasavica' and Cydomalus/'Wu Jiu Xiang', respectively.

Discussion

AFLP is considered to be the most effective method in examining genotyping of *Pyrus communis* L. and its cultivars (Monte-Corvo *et al.*, 2002). The present results showed high polymorphism and demonstrate the suitability of AFLP method for genotyping. In this study the average percentage of polymorphic fragments was 89.5%, which is higher by 2.5% than what was indicated by Monte-Corvo *et al.* (2000), which reported that the percentage of polymorphic groups within pear cultivars was 87%. This difference is probably due to the fact that the presented research included an interspecific hybrid, botanical species, rootstocks, and intergeneric hybrid *Cydomalus*.

The similarity coefficients of investigated samples ranged from 0.8998 to 0.62233. Based on these values it can be stated that the analysed genotypes can be considered as different cultivars. Cervera *et al.* (1998) stated that if the similarity coefficient between two samples is equal or higher than 0.9, one of the samples might be considered a clone of the same cultivar.

Intergeneric hybrid *Cydomalus,* which derives from *Malus domestica* Borkh and *Cydonia oblonga* Mill crossing (De Paoli, 2002) was located in a separate cluster in dendrogram and its similarity coefficient with the other tested cultivars ranged from 0.622 to a relatively high value of 0.713.

Shengua *et al.* (2002) stated that the similarity coefficient between *Pyrus communis* L. and *Pyrus betulaefolia* Bunge was measured to be 0.528, however in this study, their similarity coefficient was measured to be 0.631. This deviation is caused by the fact that the pear seedling (*Pyrus communis* L.) from this research was not a pure botanical species but a selected rootstock type pear seedling.

Challice and Westwood (1973) presented a closer relationship between *Pyrus betulaefolia* Bunge and European pear species concluding that *Pyrus betulaefolia* Bunge could be an intermediate type between European and Asian pears. Bao *et al.* (2007, 2008) found similar results using SSR and AFLP methods. In this work, the average similarity coefficient between European cultivars and *Pyrus betulaefolia* Bunge was 0.7704 and the average similarity coefficient between Asian cultivars and *Pyrus betulaefolia* Bunge was 0.768. Since this value is higher than the average similarity coefficients between European and Asian cultivars, it can be concluded that *Pyrus betulaefolia* Bunge is an intermediate type between European and Asian pears.

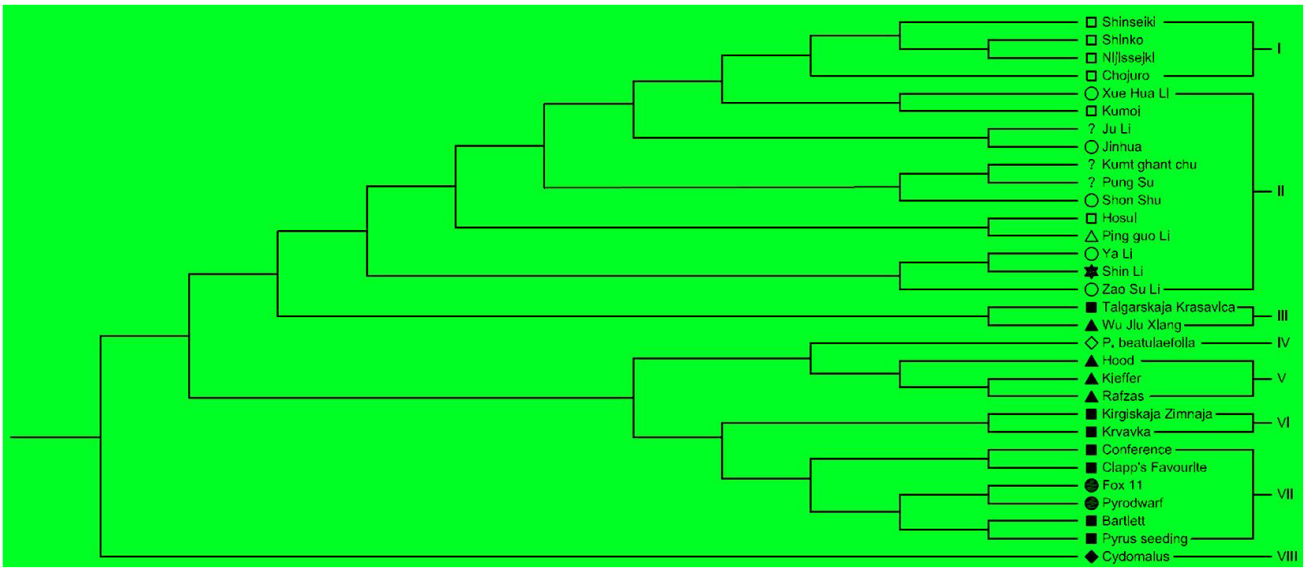

Fig. 1. Nei's genetic distance dendrogram of 31 interspecific hybrids and cultivars of pears was constructed using the UPGMA method (Bootstrap value 100). (□) Japanese pear, (○) Chinese white pear, (?) unknown cultivar, (●) rootstock, (▪) European pear, (◊) botanic species, (▲) interspecific hybrid, (♦) intergenetic hybrid, (★) Chinese sand pear, (△) Ussuriensis pear

Chinese sand pears and Chinese white pears did not cluster in the dendrogram into separate groups and not even into subgroups, but they are mixed together. This aspect is also described in many other studies (Teng *et al.*, 2002; Bao *et al.*, 2007; Bao *et al.*, 2008). Both pear types have similar pear peroxidase isozymic formula (Lin and Shen, 1983) and similar ultrastructure of pollen (Zou *et al.*, 1986). Chinese white and Chinese sand pears are very similar in morphological and pomological characters as well, which suggests a very close genetic relationship between these two types. Bao *et al.* (2008) described a close genetic relationship of Chinese sand pear cultivars with certain Chinese white pear cultivars coming from Anhui Province, which is located in the northern border of the area from where the Chinese sandy cultivars started to spread. The results from evaluation of pomological characteristics show a relatively high degree of similarity in both types of pear cultivars but there are also significant cultivar differences (Nečas *et al.*, 2016). Some differences between cultivars may occur due to different development and due to local geographic and climatic factors in isolated regions.

The origin of Japanese pear cultivars was considered to be the same as of Chinese sand pear cultivars, which derived from *Pyrus pyrifolia* (Burm.) Nak. Morphological and pomological characteristics of Japanese cultivars are very similar to Chinese sand cultivars (Teng *et al.*, 2002). Bao *et al.* (2008) disclosed that Japanese pear cultivars are in the same cluster with some Chinese sand pears and Chinese white pears, especially those that come from Chinese Zhejiang province and provinces adjacent to it. Particularly some Japanese cultivars coming from Kochi Prefecture are very similar with certain Chinese sand pear cultivars of Zhejiang Province and Fujian. Probably the most likely theory is that ancient Chinese cultivars were brought to Japan through maritime trade route that once connected the Japanese prefecture of Kochi and the Chinese province of Zhejiang (Bao *et al.*, 2007). Recent studies based on analyses of different DNA markers (Teng *et al.*, 2001, 2002; Shen *et al.*, 2006; Bao *et al.*, 2007, 2008) concluded that Japanese pear cultivars could have developed from progenitor genotypes originating in ancient China. The same results were obtained in this work, as all the Japanese cultivars clustered in the same group as the Chinese sand pears.

In group I. all Japanese pears had high similarity indexes: 'Chojuro' (chance seedling from Japan), 'Shinko' (chance seedling from open pollinated variety 'Nijisseiki'), 'Shinseiki' (crossing varieties 'Nijisseiki' × 'Chojuro'), and 'Nijisseiki' (chance seedling from Japan). These cultivars have very similar pomological characters (Nečas *et al.*, 2016) and their close relationships were confirmed in this study as well.

Group III., which is part of the third cluster of Oriental pears, consisted of interspecific hybrid 'Wu Jiu Xinag' ('Ya Li' × 'Bartlett') (Gao, 2015) and 'Talgarskaja Krasavica' (chance seedling of 'Forest Beauty'). Group V. which is part of the second cluster of European pears, consisted of 'Hood' (Asian × European pear), 'Rafzas' (*P. pyrifolia* × 'General Leclerc'), and 'Kieffer' (*P. pyrifolia* × 'Bartlett'). Breeding studies with other genera showed that from the molecular point of view, interspecific hybrids are usually located somewhere between the parental species, but after several generations of segregation they usually drift closer to one of them (Simonovik *et al.*, 2007). In the case of interspecific pears, during an interspecific recombination between cultivated and wild genotypes, the progeny probably drifted closer to the wild type, as wild cultivars have usually better chance of surviving (Sisko, 2009). The results of this study are in agreement with this knowledge and it can be assumed that cultivars 'Wu Jiu Xinag' and 'Talgarskaja Krasavica' belong to Asian pears and cultivars 'Hood', 'Rafzas', and 'Kieffer' belong to European pears.

European pear cultivars clustered in one group. The origin of all of these pears is *Pyrus communis* L. Pyrodwarf rootstock was bred from 'Old Home' and 'Bonne Louise d' Avranches' cultivar (Jacob, 1998) and Fox 11 rootstock is a progeny of an open pollinated 'Volpina' cultivar (Elkins *et al.*, 2012). Cultivar 'Krvavka' is a landrace from White Carpathians (Czech Republic). 'Kirgizkaja Zimnaja' is a cultivar with unknown origin. Its morphological and pomological characters belong to European pears, but its fruit shape is similar to Chinese white pears (Nečas *et al.*, 2016).

Tested cultivars of unknown origin were classified based on their similarity coefficient as follows: 'Ju Li' clustered in the same cluster as 'Jinhua' (0,842). 'Jinhua' and 'Ju Li' have similar morphological and pomological characters (Nečas *et al.*, 2016). According to these results 'Ju Li' is probably a Chinese white pear. Varieties 'Kumt Ghant Chu' and 'Pung Su' have the highest similarity coefficient with 'Nijisseiki' (0.85833 and 0.8827, respectively). Their morphological and pomological characters are very similar to 'Nijisseiki' (Nečas *et al.*, 2016) and represent probably Japanese pear cultivars.

Conclusions

The molecular analyses used in this study enabled the placement of some unidentified genotypes into known cultivar groups. Molecular analyses also confirmed close relationship between Chinese white, Chinese sand and Japanese pear cultivars. Interesting results were found in clustering of interspecific hybrids and *Pyrus betulaefolia* Bunge.

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