RESEARCH NOTE

AFLP fingerprinting analysis of some cultivated varieties of sea buckthorn (*Hippophae rhamnoides*)

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Introduction

The genus *Hippophae* includes 7 species and 8 subspecies in the world (Swenson and Bartish 2002; Bartish *et al.* 2002; Sun *et al.* 2002), these species are all diploid of 2n = 24 and are restricted to Qinghai-Xizang plateau and adjacent areas except Hippophae rhamnoides L. which is naturally distributed from Asia to Europe and was introduced to South and North America (Rousi 1971; Lu 1997; Heinz and Barbaza 1998; Lian *et al*. 2000; Bartish *et al*. 2000a, 2002; Roy *et al*. 2003). Sea buckthorn (*H. rhamnoides*) has shown enormous agricultural, ecological, nutritional, medical and ornamental values (Vikberg and Itamies 1999; Zadernowski *et al*. 2002; Cheng *et al*. 2003; Tsydendambael and Vereshchagin 2003). Commercially, sea buckthorn has been suggested as a hardy, multi-purpose plant with orange, red or yellow berries, and being widely introduced and extended in the world, especially in China.

H. rhamnoides ssp. *sinensis* and *H. rhamnoides* ssp. *mongolica* of six subspecies of *H. rhamnoides* are mainly and widely distributed in China, Mongolia, Russia and other countries of Asia and eastern Europe. Breeding programmes for improving adaptability, tolerance and yield have been carried out since 1960. Some fine cultivated varieties have been successfully selected or bred by scientists of these countries, especially in China, Russia and Mongolia. However, the different cultivated varieties have advantages and disadvantages. Cultivated varieties from China have strong adaptability and are fast-growing, but bear small fruits, more thorns, short fruit stalk, and have lower content of bioactive substances, and are more prone to dried-shrink disease. Cultivated varieties from Russia and Mongolia show many promising agronomic traits, such as big fruits, few or no thorns, long fruit stalk and high content of bioactive substances, and resistance to dried-shrink disease (some varieties), but show weak adaptability and are slow-growing.

Many cultivated varieties from Russia and Mongolia have been introduced into China since 1991 to supply and improve sea buckthorn germplasm of China and to gain commercial benefit. Some of them adapt very well to the new habitat and yield high economic benefit. Some of the fine cultivated varieties from Russia and Mongolia selected and bred by Chinese breeders are being planted on a large scale in China. However, sea buckthorn breeding in China also faces serious problems, such as unstable adaptability, unsteady yield and quality of introduced varieties, and difficulty of harvesting native varieties with thorns. An available approach for breeding fine varieties is crossing between *H. rhamnoides* ssp. *sinensis* and *H. rhamnoides* ssp. *mongolica*, and crosses between them once bred two fine hybrids (Liaohuyihao and Liaohuerhao) in China, but selecting parents is still a major problem. Knowledge of genetic relationships in parental varieties could improve the effectiveness of breeding programmes (Le Thierry d' Ennequin *et al.* 2000). Moreover, some of the commercially desirable sea buckthorn cultivated varieties could be reproduced by micropropagation; thus, unauthorized commercialization of patented varieties leads to lawsuits requiring careful technical investigation. However, investigators often look only at morphological characters, which do not always yield clear

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answers concerning identification of the sea buckthorn varieties. The molecular approach has proved increasingly valuable in the identification of plant varieties (Morell *et al*. 1995; Welsh and Mcclelland 1990; Williams *et al*. 1990; Congiu *et al*. 2000).

Different types of genetic markers have been used to assess phylogenetic relationships and diversity in sea buckthorn: initially, isozymes (Yao and Tigerstedt 1993), later randomly amplified polymorphic DNA (RAPD) (Bartish *et al*. 1999; 2000; Bartish *et al*. 2000a; Chowdhury *et al*. 2000; Ruan *et al*. 2004), and, recently, chloroplast DNA and internal transcribed spacer (ITS) seqnences (Bartish *et al*. 2002; Sun *et al*. 2002). Yao and Tigerstedt (1993) looked at isoenzymes and found a within-population genetic diversity of 0.168. They also noted that the populations of *H. rhamnoides* ssp. *rhamnoides* were more diverse (as revealed by Nei's genetic distance of 0.037) than those of *H. rhamnoides* ssp. *sinensis* (0.007). Bartish *et al* (1999) studied 10 populations of *H. rhamnoides* ssp. *rhamnoides* from Northern Europe using RAPD and estimated that 85% of the genetic variation was within the populations and 15% due to differences among the populations. This result was supported by further RAPD analysis by Bartish *et al.* (2000b).

Both RAPD and AFLP markers are useful for studying relationships among DNA sequences, independently of their location in the chromosomes or of particularities in their nucleotide sequence (Cabrita *et al*. 2001). However, the higher multiplex ratio of AFLP markers makes them more suitable for distinguishing between closely related genotypes, such as different clones within a given cultivar (Cervera *et al*. 1998), which often, having diverged by small mutational events, present minimal genetic differences (Cabrita *et al*. 2001). AFLP markers have been used to study genetic relationships in many plant species, such as *Setaria italica* and *S. viridis* (Le Thierry d' Ennequin *et al.* 2000), *Gossypium* (Abdalla *et al*. 2001), *Ficus carica* (Cabrita *et al*. 2001), *Saccharum* spp. (Lima *et al*. 2002) and *Olea europaea* (Sensi *et al.* 2003).

Though we evaluated genetic relationships among 14 sea buckthorn cultivated varieties from China, Russia and Mongolia using RAPD markers earlier (Ruan *et al*. 2004), in this study we further report the usefulness of AFLP markers for analysing DNA fingerprint patterns and genetic relationships among 15 sea buckthorn cultivated varieties from China, Russia and Mongolia, which are mainly and widely planted and extended varieties in China. The data provides a scientific basis for crossing strategies for sea buckthorn, identification of cultivated varieties, and management of germplasm.

Materials and methods

Plant materials and DNA extraction

The 15 sea buckthorn cultivated varieties used in this study, about 4–5 years old, are growing in the YCIT (Institute of Yancheng Technology) test garden, Yancheng city, Jiangsu Province of China. Nine cultivated varieties of *H. rhamnoides* ssp. *mongolica* from Russia and Mongolia were: Xiangyang (XY), accession no. YCXY02009; Chengse (CS), YCCS02017; Chuyi (CY), YCCY02033; Aleiyi (ALY), YCALY02141; Zeliang (ZL), YCZL02084; Huoguang (HGG), YCHGG02145; Nuyou (NY), YCNY-02023; Hongyun (HY), YCHY02187; and Wulangemu (WLGM), YCWLGM02255. Three cultivated varieties of *H. rhamnoides* ssp. *mongolica* from China were: Zhongguoshaji (ZGSJ), YCZGSJ02024; Zhongguoyou (ZGY), YCZGY02251; and Hongguo (HG), YCHG02243. Three hybrids from China were: Liaohuyihao (LHYH), YCLHYH-02006; Liaohuerhao (LHEH), YCLHEH02001; and Zajiaoliangzhong (ZJLZ), YCZJLZ02076. The source and morphological characteristics of 14 sea buckthorn cultivated varieties were described by Ruan *et al.* (2004). The other, ZJLZ, is a hybrid of ZGY × wild *H. rhamnoides* ssp. *sinensis* with the morphological characteristics of few or no thorns, medium fruit size and fast growth. Three hundred annual micropropagated seedlings of ZJLZ were introduced from the experimental field at Ansai County of Shaanxi Province in 1999 and planted in YCIT test garden.

Total genomic DNA was isolated from fresh leaf tissue of 4–5-year-old sampling plants as described by Ruan *et al.* (2004).

AFLP analysis

AFLP analysis was performed using Analysis System II (GIBCO-BRL Life Technologies) according to the manufacturer's protocol. Genomic DNA (350 ng) was digested with 2.5 U *Eco*RI and 5 U *Mse*I (MBI, Fermentas, Lithuania) in a final volume of 25 µl. After inactivation (15 min at 72°C), two different adaptors, for *Eco*RI and *Mse*I, were ligated to the ends of the genomic restriction fragments. The digested and ligated template DNA was pre-amplified using EcoRI + 1 (5′-GACTGCGTACCAA-TTCA-3′) and *Mse*I + 1 (5′-GATGATGCCTGAGTAAC-3′) primers in a total volume of 50 µl containing 5 µl of ligation mixture (diluted 10 times in TE). The cycle profile was as follows: 94°C incubation for 30 s, then cycled 30 times (denaturation 94°C for 30 s, annealing 56°C for 1 min, extension 72°C for 1 min). Selective amplification was performed in a 50-µl final volume containing 5 µl of pre-amplification products (diluted 50 times in TE) with the following cycling profile: 13 cycles with annealing temperatures decreasing by 0.7°C each cycle starting with 94°C for 30 s, 65°C for 30 s, 72°C for 1 min; and ending with 27 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 31 min. The selective amplification primer combinations had an extension of three additional nucleotides (see table 1). In all reactions, only the *Eco*RI primers were 5′ labelled with ^{33}P. Primer selection was

performed on four varieties of the samples using eight *Eco*RI and eight *Mse*I primers for a total of 64 combinations in a Perkin-Elmer 2700 thermocycler. Based on the number and quality of polymorphic fragments, eight primer combinations (see table 1) were selected and selective amplification was carried out on all samples. Amplification products were separated on a 6% polyacrylamide gel, and visualized by exposing an X-ray film to the dried gel.

A reproducibility assay was performed to increase the consistency of the results. Two selected cultivated varieties (ZGSJ and ZL) were independently processed from the beginning of the AFLP analysis five times with two different primer combinations (E + AAG/M + CAG and E + AGG/M + CTA).

Data analysis

The AFLP fragments were scored as present (1) or absent (0). Jaccard (1908) coefficient was calculated by using $NTSYS_{2.02}$ (Rohlf 2000). Similarity tree was produced based on the Jaccard coefficient with the unweighted combination group method using arithmetic averages (UPGMA) and the SAHN clustering program. The goodness of fit of the clustering was tested using the MXCOMP program, which directly compares the original similarity matrix and the cophenetic value matrix. Cluster analysis and MXCOMP were conducted by using $NTSYS_{2.02}$ (Rohlf 2000).

Results and analysis

AFLP analysis

The results obtained for the eight different primer combinations used in this study are shown in table 1. A total of 731 AFLP bands were detected using eight combinations of primers, 645 of which were polymorphic, representing 88% of the total number of bands. The primer combinations differed in their ability to detect bands in the 15 sea buckthorn cultivated varieties. The number of polymorphic bands over all samples detected by individual primer combinations ranged from 47 for primer combination E + ACG/M + CTA to 107 for primer combination E + ACC/M + AAT (table 1). The number of unique bands over all samples detected by individual primer combinations ranged from one for primer combination E + ACG/M + CTA to 28 for E + AGG/M + CAG, with a mean of 16. Eight primer combinations generated 128 unique bands (18%) (table 1).

Numbers of bands detected by eight primer combinations ranged from 267 (LHEH) to 449 (ZJLZ), with a mean of 340, and unique bands varied from 1 (HG, HGG and LHEH) to 43 (ZJLZ), with a mean of 8.5 (table 1).

The reproducibility test, shown in figure 1, carried out with primer combination E + AAG/M + CAG and E + AGG/M + CTA, appeared to be highly consistent.

Cluster analysis

Genetic similarities (calculated with Jaccard coefficient) among the 15 sea buckthorn cultivated varieties ranged from 0.29 (between ZGSJ and WLGM) to 0.78 (between CS and HGG), with a mean of 0.48. Based on the Jaccard coefficient, a UPGMA dendrogram was constructed (figure 2). The measure of goodness of fit of cluster analysis was given by the 'cophenetic correlation' (r) calculated with the MXCOMP program. The dendrogram had a ma-

Table 1. DNA fingerprinting patterns of 15 sea buckthorn cultivated varieties based on AFLP markers.

	Number of bands (total bands, and in parcentheses, unique bands)								
Variety	E + AAC/M + CAA[a] (115, 95, 83, 19)[b]	E + AAG /M + CAG (72, 58, 81, 16)	E + ACT/ M + CAT (93, 80, 86, 16)	E + ACC/ M + CAT (116, 107, 92, 16)	E + ACG /M + CTA (51, 47, 92, 1)	E + AGG /M + CAC (100, 93, 93, 17)	E + AGG/M + CAG (91, 77, 85, 28)	E+AGG/ M+ CTA (93, 88, 95, 15)	Sum (731, 645, 89, 128)
ZGSJ	65 (1)	33 (2)	32 (0)	37 (0)	30 (1)	26 (3)	32 (0)	67 (7)	322 (14)
LHEH	59 (0)	36 (0)	46 (0)	33 (0)	18 (0)	43 (1)	24 (0)	8 (0)	267 (1)
NY	53 (0)	38 (0)	61 (1)	49 (1)	18 (0)	43 (0)	32 (2)	57 (0)	351 (4)
ALY	73 (0)	31 (0)	52 (0)	39 (1)	4 (0)	32 (0)	34 (1)	40 (0)	271 (2)
HY	76 (1)	37 (2)	63 (3)	60 (3)	14 (0)	48 (4)	49 (4)	55 (0)	402 (17)
ZL	47 (0)	42 (1)	44 (2)	27 (0)	25 (0)	45 (4)	50 (10)	42 (0)	322 (17)
HG	57 (0)	38 (0)	51 (0)	48 (0)	48 (0)	32 (0)	39 (1)	15 (0)	328 (1)
XY	89 (11)	38 (0)	71 (5)	54 (1)	19 (0)	25 (0)	31 (0)	48 (1)	375 (18)
WLGM	69 (1)	25 (0)	43 (0)	39 (0)	41 (0)	55 (1)	36 (0)	64 (1)	372 (3)
HGG	61 (0)	31 (0)	50 (0)	33 (1)	15 (0)	40 (0)	29 (0)	61 (0)	320 (1)
CS	70 (0)	33 (0)	56 (0)	39 (0)	17 (0)	42 (1)	27 (0)	55 (1)	339 (2)
ZJLZ	68 (4)	50 (9)	58 (5)	96 (9)	18 (0)	60 (3)	45 (8)	54 (5)	449 (43)
LHYH	69 (0)	30 (1)	50 (0)	32 (0)	39 (0)	24 (0)	33 (1)	60 (0)	337 (2)
ZGY	68 (0)	31 (1)	53 (0)	33 (0)	18 (0)	24 (0)	36 (0)	50 (0)	313 (1)
CY	64 (1)	31 (0)	58 (0)	40 (0)	23 (0)	31 (0)	33 (1)	48 (0)	328 (2)

[a]Primer combinations.

[b]The numbers are: total number of bands, number of polymorphic bands, polymorphism rate (%), and number of unique bands.

ZGSJ: Zhongguoshaji; LHEH: Liaohuerhao; NY: Nuyou; ALY: Aleiyi; HY: Hongyun; ZL: Zeliang; HG: Hongguo; XY: Xiangyang; WLGM: Wulangemu; HGG: Huoguang; CS: Chengse; ZJLZ: Zajiaoliangzhong; LHYH: Liaohuyihao; ZGY: Zhongguyou; CY: Chuyi.

trix correlation $r = 0.864$, which was interpreted as a good fit.

At a level of 0.47 (Jaccard coefficient), the following clusters were formed: cluster I included four cultivated varieties (*H. rhamnoides* ssp. *mongolica*) from Russia (XY, CY, HGG and CS), one (*H. rhamnoides* ssp. *mongolica*) from Mongolia (WLGM), one (*H. rhamnoides* ssp. *sinensis*) from China (HG), and three hybrids (LHYH, LHEH and ZJLZ) from China; cluster II included four cultivated varieties from Russia (ALY, NY, HY and ZL), which all originated from *H. rhamnoides* ssp. *mongolica.* The other two cultivars (*H. rhamnoides* ssp. *sinensis*) from China (ZGSJ and ZGY) were not grouped at that level. At a level of 0.60, one subgroup within cluster I was resolved, including XY, CY, WLGM, HGG, CS and LHYH; and one subgroup within cluster II also was resolved, including NY, HY and ALY. At a level of 0.66, two sub-subgroups within the subgroup of cluster I were resolved, one including XY and CY, another consisting of WLGM, HGG, CS and LHYH; within the later, HGG was very closely related to CS, and the two formed a subgroup with LHYH. Two sub-subgroups within the subgroup of cluster II also were resolved, one including NY and HY, and another only ALY.

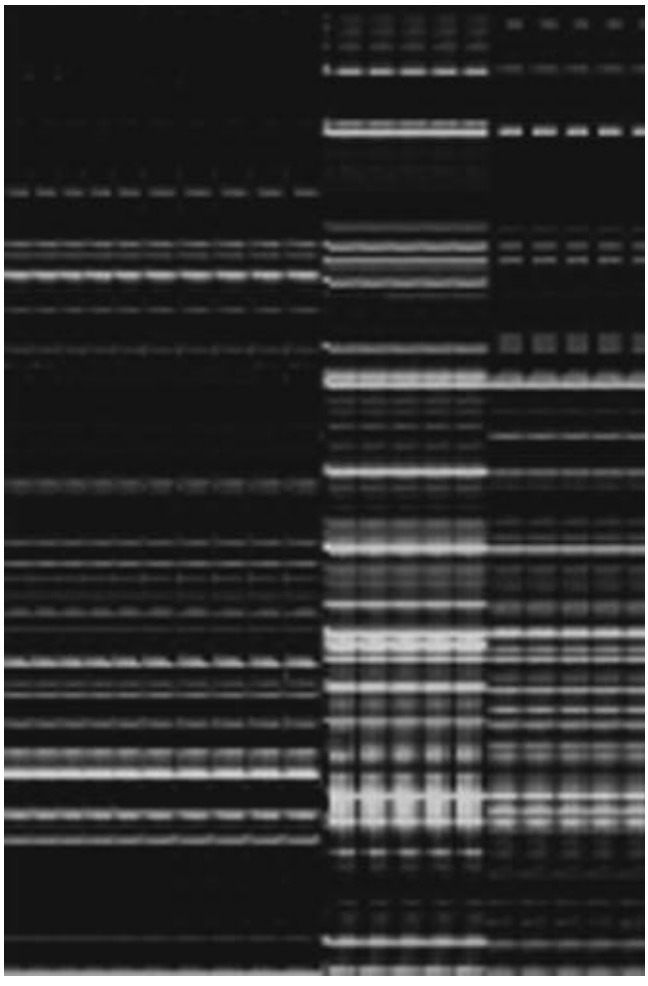

Figure 1. Digitalized electrophorogram of AFLP profile of genomic DNA. The figure represents the reproducibility assay performed on ZGSJ (lanes 1–5) and ZL (lanes 6–10) using primer combination E + AAG/M + CAG, and ZGSJ (lanes 11–15) and ZL (lanes 16–20) using primer combination E + AGG/M + CTA.

Discussion

Fifteen sea buckthorn cultivated varieties from China, Russia and Mongolia were fingerprinted with AFLP markers. The fingerprinting patterns obtained allowed unequivocal identification of each cultivated variety. Some primer combinations were better suited to discriminate among the varieties (table 1). Primer combination E + CC/M + AT detected the highest number of polymorphic loci in all the varieties. It is followed by primer combinations E + AC/M + AA and E + GG/M + TA.

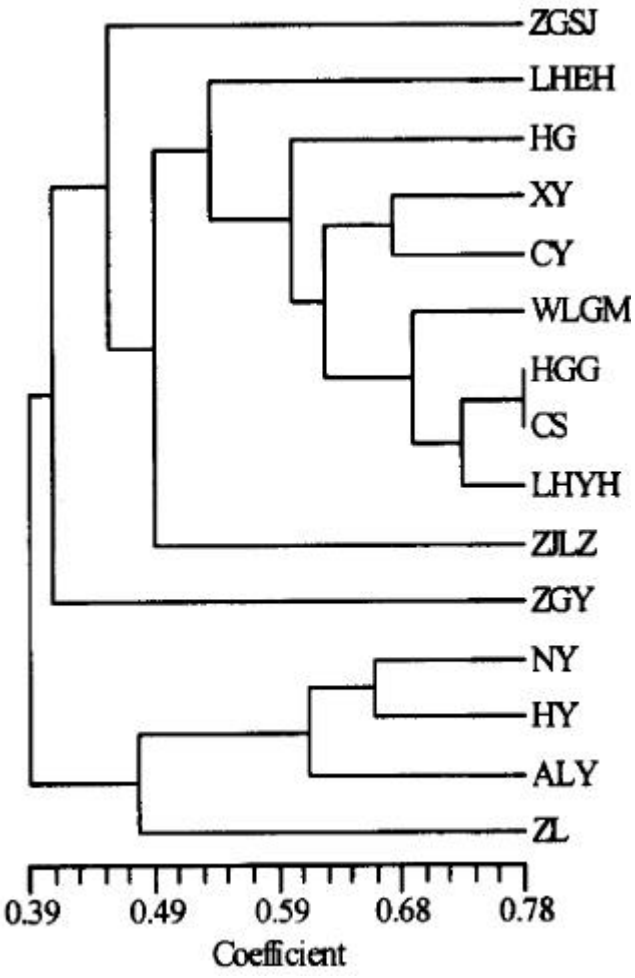

Figure 2. Dendrogram of 15 sea buckthorn varieties by UPGMA cluster analysis based Jaccard coefficient, using 645 AFLP polymorphic bands obtained by eight primer combinations.

Genetic similarity between ZGSJ and WLGM was the smallest (0.29), as was expected since they represent different subspecies. ZJLZ is a hybrid of ZGY × wild *H. rhamnoides* ssp. *sinensis;* however, the AFLP results showed it to be distinct from ZGY and ZGSJ.

ZGY was selected from wild *H. rhamnoides* ssp. *sinensis* by Chinese scientists breeders. However, the genetic similarities based on AFLP (0.333) and RAPD (0.460; see Ruan *et al*. 2004) data were lower between ZGY and ZGSJ, and the clustering results with AFLP and RAPD markers (see Ruan *et al*. 2004) showed it to be distinct from ZGSJ. Based on these, we infer that it may be incorrect to see ZGY as originating from wild *H. rhamnoides* ssp. *sinensis*. ZGY was selected from the interzone of natural distribution of wild *H. rhamnoides* ssp. *sinensis* and wild *H. rhamnoides* ssp. *mongolica*, and its morphological characteristics are intermediate, such as wide leaf, no or few thorns and fastgrowth. These suggest that ZGY may be a hybrid between wild *H. rhamnoides* ssp. *sinensis* and wild *H. rhamnoides* ssp. *mongolica*.

At Jaccard coefficient 0.59, HG was grouped together with cultivated varieties of *H. rhamnoides* ssp. *mongolica.* HG was selected from wild *H. rhamnoides* ssp. *sinensis* distributed in Inner Mongolia of China. It is difficult to distinguish HG from HY (*H. rhamnoides* ssp. *mongolica*) by phenotypic characteristics, and the region where it was selected borders on the distribution zone of wild *H. rhamnoides* ssp. *mongolica*. These indicated that introgression is likely to have taken place between HG and wild *H. rhamnoides* ssp. *mongolica*. However, we cannot accurately determine the genetic origin of HG.

While LHYH and LHEH are hybrids of Qiuyesike (*H. rhamnoides* ssp. *mongolica*) × *H. rhamnoides* ssp. *sinensis*, AFLP analysis showed that the two grouped with cultivated varieties from Russia and Mongolia (*H. rhamnoides* ssp. *mongolica*) at Jaccard coefficient 0.53. It may be from matroclinal inheritance, or perhaps in the long term of artificial selection matroclinous variations were selected. Matroclinous characteristics include big fruits, few or no thorns and low susceptibility to dried-shrink disease.

The dendrogram obtained with AFLP markers is in agreement, except for ZJLZ, with that based on RAPD markers (based on Nei and Li's genetic distance) of Ruan *et al.* (2004) to a large extent. However, RAPD gave a slightly different cluster with HG, HGG and ZL. HGG was placed in a subgroup with XY, CY, WLGM, CS and LHYH based on AFLP data but RAPD data. ZL was placed in cluster II (at a level of 0.47) with AFLPs but RAPDs. HG was grouped with XY, CY, WLGM, CS and LHYH (at a level of 0.59) with AFLPs but RAPDs. We cannot rule out the possibility that if more of primers well used in RAPD analysis one might be able to place HG, HGG and ZL more precisely in the tree.

In conclusion, our results demonstrate that AFLP markers are useful for fingerprinting and detecting genetic relationships among sea buckthorn cultivated varieties from China, Russia and Mongolia. The results can be used as guidelines for improving germplasm collection and breeding. For example, ALY has excellent adaptability to a large region of China (Shannxi, Liaoling, Helongjiang and Jiangsu province, etc.), and has promising characteristics (e.g. big fruits, high Vitamin C content in seeds and leaves, etc.). Genetic similarities between ALY and ZGSJ based on AFLP data was relatively low (0.364). Crossing between ALY and ZGSJ may breed a fine hybrid.

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