Pak. J. Bot., 55(5): 1763-1768, 2023. DOI: http://dx.doi.org/10.30848/PJB2023-5(29)

ANALYSIS OF THE GENETIC DIVERSITY OF 58 BLUE HONEYSUCKLE (*LONICERA CAERULEA* L.) GERMPLASM RESOURCES USING SRAP MARKERS

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Abstract

Blue honeysuckle is a shrub species widely distributed in the northern regions of the world, the nutritious fruits of which have high economic value and are considered to have beneficial health effects. To gain a better understanding of the genetic background of blue honeysuckle, we employed sequence-related amplified polymorphism (SRAP) markers to analyse the genetic diversity of 58 blue honeysuckle germplasms, which are naturally distributed in China, Russia, and Japan. A total of 20 SRAP primer pairs showing clear polymorphisms, screened from 90 assessed primer pairs, were used to analyse genetic relationships among the 58 blue honeysuckle germplasms. The results revealed that the 20 primer pairs amplified 172 bands, among which, 143 bands were polymorphic, with a polymorphic rate of 83.1%. Each primer pair yielded between five and 12 bands, with an average band number of 8.6. Unweighted pair-group method with arithmetic (UPGMA) cluster analysis of the SRAP marker data revealed that genetic similarity coefficients of the 58 germplasms ranged from 0.68 to 0.95. The germplasms were divided into seven groups with a genetic similarity coefficient 0.74, with germplasms from China being characterised by greater differences than those from Russia. Wild genotypes from Mount Changbai and the Great Khingan mountains were classified into a single group, indicating that cultivar classification has been based on genotype composition. In general, blue honeysuckle has a high genetic diversity, which will provide a valuable basis for future breeding.

Key words: Blue honeysuckle; Germplasm resources; Genetic diversity; SRAP.

Introduction

Blue honeysuckle (*Lonicera caerulea* L.), also referred to haskap in Japan, Canada, and other countries is a perennial deciduous shrub in the genus *Lonicera* and family Caprifoliaceae (Hummer, 2006). *L. caerulea* is generally found in cold climate regions north of latitude 45°, with the species widely distributed in the Russian Far East, northeast China, Hokkaido Island, Japan, and the southern region of North Korea. Blue honeysuckle plants are characterised by high tolerance to low temperatures, with branches and buds surviving at temperatures as low as -50 during dormancy (Huo & Qin, 2012). Consequently, the plant has often been used as a material for plant cold tolerance experiments (Imanishi *et al.*, 1998). The fruits of blue honeysuckle are noted for their abundant nutrient content, including amino acids, vitamins, mineral elements, and anthocyanins, and the products derived from these fruits, including juices, wines, jams, and canned fruit, are particularly popular (Celli *et al.*, 2014). Moreover, these fruits are also used as raw materials for the extraction of natural edible pigments, which have broad potential applications (Vasantha *et al.*, 2018).

However, despite the promising utility of blue honeysuckle, there is currently comparatively little information available regarding the plant's genetic background. The existing germplasms of blue honeysuckle are a consequence of the post-glacial migration of ancestral populations, which under the long-term influence of different soil, temperature, light, and climatic conditions, have diversified to give rise to the range of morphological variants of the species seen today (Huo *et al.*, 2005). Based on morphological, ecological, and geographical diversity, blue honeysuckle can be classified into 16 or 17 discrete species (Pojarkova, 1958). Furthermore, based on chromosomal analyses of wild populations in different regions (Plekhanova *et al.*, 1992), in conjunction with morphological (Plekhanova, 1989), anatomical (Plekhanova & Rostova, 1994), chemical classification (Plekhanova *et al.*, 1998), hybridisation (Plekhanova, 1987), and isoenzyme (Plekhanova *et al*., 1989) analyses, Plekhanova and colleagues have summarised the different types of blue honeysuckle distributed in Europe and Asia as four species and seven subspecies. Therefore, the large morphological variation between the populations, due to the correlation of blue honeysuckle species is not strong. Furthermore, in common with pea (Espósito *et al.*, 2007) and *Salvia miltiorrhiza* (Peng *et al.*, 2014) the genetic relationships among blue honeysuckle species are notably complex and are accordingly difficult to establish precisely based solely on morphological, agronomic, and anatomical methods.

Although classical approaches based on morphology, physiology, anatomy, and embryology have traditionally been used to assess plant genetic variability, over the past three decades, these aspects have increasingly been complemented by developments in molecular techniques, notably the use of molecular markers. The application of such markers provides a credible means of detecting genetic variation independently of the influence of environmental factors. (Kumar, 1999; Linda *et al.*, 2009). However, although molecular markers such as simple sequence repeats (SSRs) and random amplified

polymorphic DNA (RAPD) have been applied to analyse the relationships and genetic diversity of different blue honeysuckle taxa (Donatas *et al.*, 2014), there is as yet no generally accepted consensus regarding the taxonomic status of blue honeysuckle, owing to its complex genetic background.

In this context, sequence-related amplified polymorphism (SRAP) is a relatively new type of molecular marker that can be used to detect open-reading frame sequences based on PCR reactions (Li & Quiros, 2001). The SRAP marker technique has multiple advantages, including high reproducibility, polymorphism, simplicity, generality, and uniform distribution, and has been widely applied in genetic linkage map construction, the comparative genetics of germplasms, crop genetic diversity and phylogenetic analysis, the molecular mapping and cloning of important characters, and fingerprint development for different cultivars (Neil *et al*., 2009; Uzun *et al.*, 2009; Zhou *et al.*, 2010; Ashwini *et al.*, 2021; Zhou *et al.*, 2021). Compared with other PCR-based markers, SRAP markers are considered more useful from the perspective of analysing the genetic diversity of plants (Budak *et al.*, 2004).

In this study, we sought to assess the utility SRAP molecular markers in analysing the relationships among different blue honeysuckle germplasms, and in doing so, enhance our understanding of the genetic background of this plant and contribute to the assessment and application of blue honeysuckle germplasms and selection of superior germplasms. We believe that the findings of this study will provide a valuable basis for blue honeysuckle breeding and cultivar identification.

Materials and Methods

Plant materials: In this study, we analysed the genetic diversity of 58 naturally distributed blue honeysuckle germplasms; their geographical origins are listed in Table 1. Specimens of all 58 germplasms were raised in the germplasm nursery of the Northeast Agricultural University under natural conditions. Healthy young leaves were collected from representative plants, immediately frozen in liquid nitrogen, and then stored in a freezer at -80℃.

DNA extraction: Total DNA was extracted from leaf samples using a modified CTAB method (Guo *et al.*, 2012); the quantity and quality of the extracted DNA were determined by spectrophotometry and 0.7% (w/v) agarose gel electrophoresis, respectively. All DNA samples thus obtained were diluted to 20 ng/μL using RNA-free water and stored at -20℃.

Table 1. The blue honeysuckle germplasm resources used in this study.

No.	Origin	Germplasm	No.	Origin	Germplasm
1.	RUS	Berel	30.	RUS	Pallasii No.2
2.	RUS	Blue bird	31.	RUS	Pallasii No.3
3.	RUS	Blue spindle	32.	RUS	Pallasii No.4
4.	CHN (HLJ)	Mohe Wild	33.	RUS	Pallasii No.5
5.	CHN	L1	34.	CHN (HLJ)	Yichun No.1
6.	CHN	L2	35.	CHN (HLJ)	Yichun No.2
7.	CHN	L3	36.	CHN (HLJ)	Yichun No.3
8.	CHN (XJ)	Altay No.1	37.	CHN (HLJ)	Yichun No.4
9.	CHN (XJ)	Altay No.2	38.	CHN (HLJ)	Yichun No.5
10.	CHN (XJ)	Altay No.3	39.	CHN (HLJ)	Shangzhi No.1
11.	CHN (XJ)	Altay No.4	40.	CHN (HLJ)	Shangzhi No.2
12.	CHN (XJ)	Altay No.5	41.	CHN (HLJ)	Shangzhi No.3
13.	RUS	E3	42.	CHN (HLJ)	Shangzhi No.4
14.	RUS	E6	43.	CHN (HLJ)	Shangzhi No.5
15.	CHN (JL)	Changbai Wild Optimized	44.	CHN (HLJ)	Boli No.1
16.	JPN	Sakhalin No.1	45.	CHN (HLJ)	Boli No.2
17.	JPN	Sakhalin No.2	46.	CHN (HLJ)	Boli No.3
18.	JPN	Sakhalin No.3	47.	CHN (HLJ)	Boli No.4
19.	JPN	Sakhalin No.4	48.	CHN (HLJ)	Boli No.5
20.	JPN	Sakhalin No.5	49.	CHN (HLJ)	Changbai Wild No.1
21.	JPN	Sakhalin No.6	50.	CHN (JL)	Changbai Wild No.2
22.	RUS	VIR	51.	CHN (JL)	Changbai Wild No.3
23.	RUS	Unknown	52.	CHN (JL)	Changbai Wild No.4
24.	RUS	Vladivostok No.1	53.	CHN (JL)	Changbai Wild No.5
25.	RUS	Vladivostok No.2	54.	CHN (HLJ)	Great Khingan Wild No.1
26.	RUS	Vladivostok No.3	55.	CHN (HLJ)	Great Khingan Wild No.2
27.	RUS	Vladivostok No.4	56.	CHN (HLJ)	Great Khingan Wild No.3
2.	RUS	Vladivostok No.5	57.	CHN (HLJ)	Great Khingan Wild No.4
29.	RUS	Pallasii No.1	58.	CHN (HLJ)	Great Khingan Wild No.5

Note: CHN, China; RUS: Russia; Japan: JAP; HLJ: Heilongjiang province; XJ: Xinjiang province; JL: Jilin province

Primer selection: Based on the primers reported by Ferriol *et al*., (2003), we established 90 primer pairs by randomly combining nine forward primers (Me1 to Me9) with 10 reverse primers (Em1 to Em10). These primers, the sequences of which are listed in Table 2, were synthesised by Sangon Biological Engineering Technology and Service Co., Ltd (Shanghai, China). The 90 primer pairs were screened using blue honeysuckle germplasm from Berel, Sakhalin, and Great Khingan Wild, and 20 primer pairs amplifying clear polymorphic products were selected for use in further analyses (Table 3).

The SRAP-PCR reaction system: The 20-μL PCR reaction systems consisted of 2.0 μL of 10× PCR buffer, 1.0 U Tag DNA polymerase, 20 ng DNA template, 0.20 mmol/L dNTPs, 2.0 mmol/L Mg^{2+}, and 0.3 μmol/L primer. The amplification was performed using the following program: initial denaturation at 94℃ for 5 min; five cycles of denaturation at 94℃ for 1 min, annealing at 35℃ for 1 min, and extension at 72℃ for 90 s; 35 cycles of denaturation at 94℃ for 1 min, annealing at 50℃ for 1 min, and extension at 72℃ for 90 s; a final extension at 72℃ for 8 min; and then maintained at 4℃. The PCR products were resolved electrophoretically on 1.8% agarose gels in l× TAE buffer at an electrical field strength of 5 V/cm, followed by GoldView staining and image acquisition using an Alphalmager HP gel imaging system.

Statistical analysis: Data conversion for electrophoretic bands was conducted using a 1/0 classification. For the statistical analysis of bands, clear and repeatable bands were recorded as 1 and the absence of expected (non-amplified) bands was recorded as 0. Similarity coefficients were calculated using NTsys–Pcversion 2.1 software (Rohlf, 2000). A dendrogram was drawn using the unweighted pair-group method with arithmetic means (UPGMA).

Results

Polymorphism analysis based on SRAP amplification: The electrophoretic bands produced by amplifying with primer pair Me1/Em6 are shown in Fig. 1. The bands were clear and dense, with sizes ranging from 100 to 2000 bp. As shown in Table 3, amplification with the 20 selected primer pairs yielded a total of 172 bands, with each of the primer pairs amplifying between five and 12 bands (average of 8.6 bands). Among these 172 bands, 143 were polymorphic, with a polymorphism rate of 83.1%. Primer pair Me1/Em3 produced the largest number (12) of bands, whereas the Me4/Em4 pair produced the least number (5). Of the 172 bands amplified, six bands were found to be commonly amplified for all screened germplasms, thereby implying a degree of homology among these germplasms.

Table 2. Sequences of the SRAP primers used in this study.

Code	Forward primer (5`-3`)	Code	Reverse primer (5`-3`)
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAAACCGGAGC	Em2	GACTGCGTACGAATTTGC
Me3	TGAGTCCAAACCGGAAT	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAAACCGGACC	Em4	GACTGCGTACGAATTTGA
Me5	TGAGTCCAAACCGGAAG	Em5	GACTGCGTACGAATTAAC
Me6	TGAGTCCAAACCGGTAA	Em6	GACTGCGTACGAATTGCA
Me7	TGAGTCCAAACCGGTCC	Em7	GACTGCGTACGAATTCAA
Me8	TGAGTCCAAACCGGTGC	Em8	GACTGCGTACGAATTCTG
Me9	TGAGTCCAAACCGGTAG	Em9	GACTGCGTACGAATTCGA
		Em10	GACTGCGTACGAATTCAG

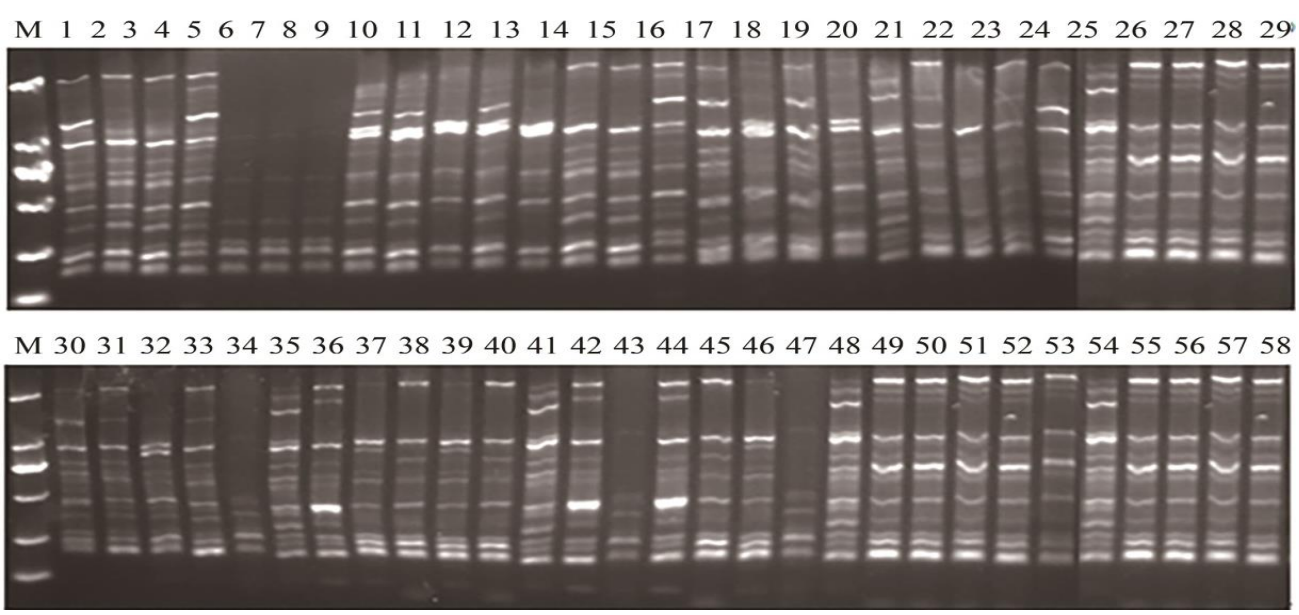

Fig. 1. SRAP amplification patterns for the genomic DNA of 58 *Lonicera caerulea* germplasms using the primer pair Me1/Em6.

Table 3. The 20 selected pairs of polymorphic primers and the polymorphisms obtained in SRAP analysis.

Primer pairs	Total bands	Polymorphic bands	Polymorphic rate/%
Me2/Em1	8	8	100
Me5/Em1	6	6	100
Me8/Em1	11	11	100
Me2/Em2	7	7	100
Me9/Em2	8	7	87.5
Me1/Em3	12	12	100
Me4/Em4	5	5	100
Me4/Em5	9	8	88.9
Me1/Em6	11	7	63.6
Me2/Em6	10	7	70
Me6/Em6	12	7	58.3
Me2/Em7	7	6	85.7
Me3/Em7	8	8	100
Me 4/Em7	8	6	75
Me5/Em7	9	7	77.8
Me3/Em8	8	6	75
Me3/Em9	6	6	100
Me4/Em10	8	7	87.5
Me6/Em10	11	7	63.6
Me9/Em10	8	5	62.5

Cluster analysis of blue honeysuckle germplasms: Using the SRAP amplification data, we performed UPGMA cluster analysis to examine the relationships among the 58 blue honeysuckle germplasms (Fig. 2). The genetic similarity coefficient of the 58 germplasms was within the range of 0.53–0.96, and at a genetic similarity coefficient of 0.74, the germplasms were classified into seven groups. Group 1 consisted of the germplasms Berel, Blue bird, Blue spindle, E3, E6, and VIR; Group 2 consisted of Mohe Wild germplasms from Heilongjiang (China), Changbai Wild Optimized from Jilin (China), Pallasii, Sakhalin, Vladivostok, and unknown germplasms No. 23; Group 3 consisted of germplasms from Boli, Yichun, and Shangzhi (Heilongjiang, China); Group 4 consisted of germplasms from Altay (Xinjiang, China). Group 5 consisted of Great Khingan Wild germplasms from Heilongjiang (China). Group 6 consisted of Changbai Wild germplasms; and group 7 consisted of hybrids.

Principal coordinates analysis of blue honeysuckle germplasms: A principal coordinates analysis of the 58 blue honeysuckle germplasms was conducted based on genetic similarity coefficients, using NTSY2.1 software. As shown in Fig. 3, germplasms with a greater similarity coefficient, and hence shorter genetic distance between each other, have closer relationships. The results obtained for principal coordinates analysis were generally similar to those of cluster analysis, although some germplasms were classified into the same group, which can plausibly be attributed to the different grouping methods. Principal coordinates analysis can, nevertheless, directly and clearly show the relationships among different blue honeysuckle germplasms.

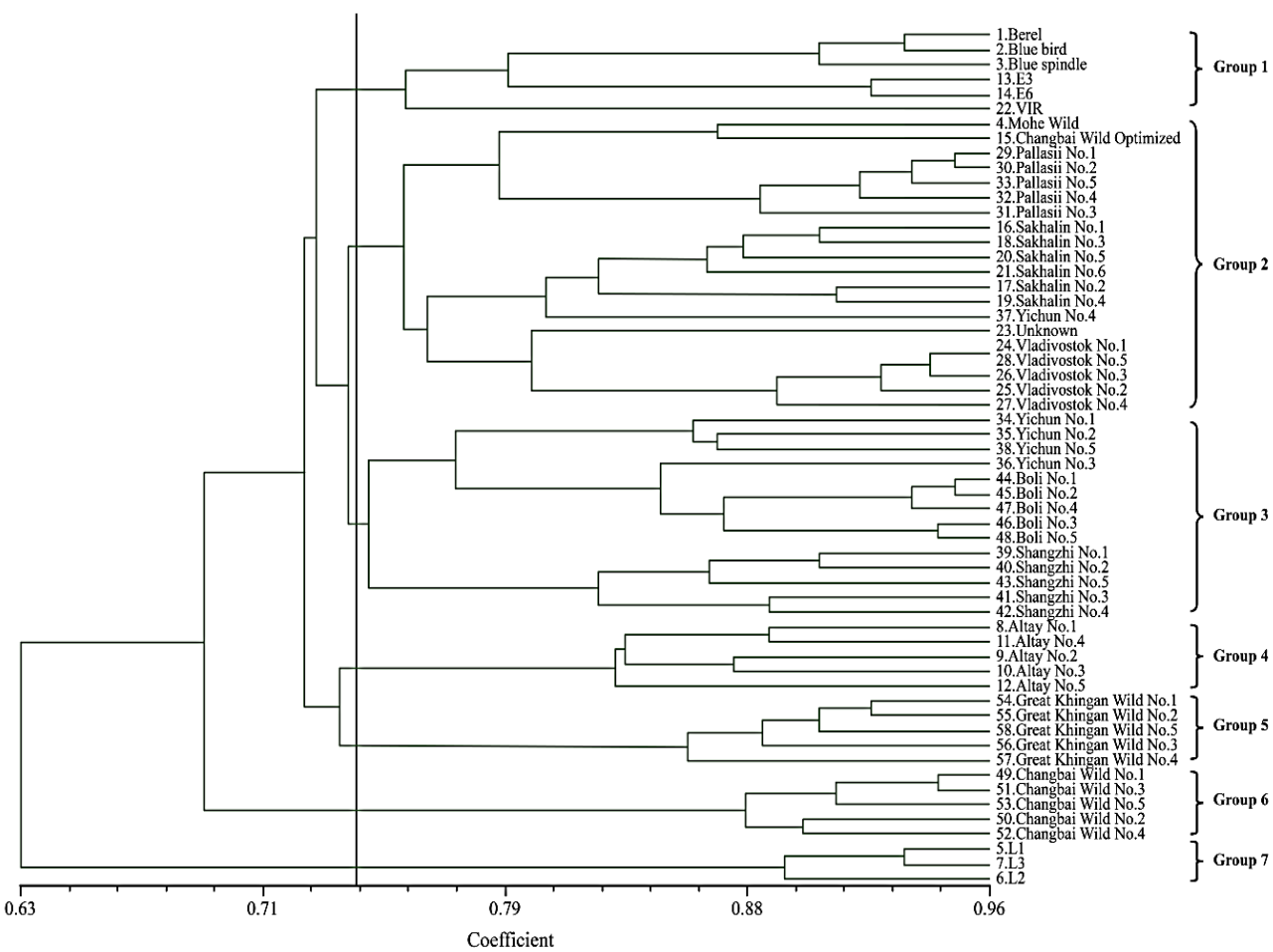

Fig. 2. A dendrogram of 58 germplasms based on UPGMA cluster analysis of the SRAP amplification data.

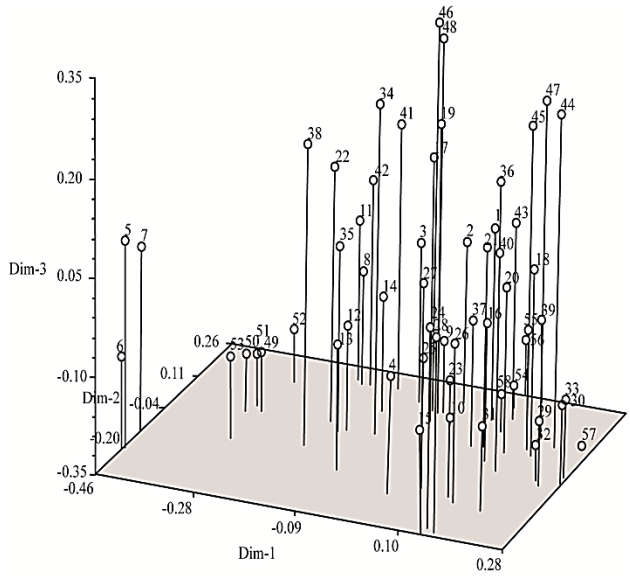

Fig. 3. Diagram of the relationships among 58 germplasms based on principal coordinates analysis (codes used for germplasms are shown in Tables 2-2).

Discussion

The available literature describing the genetic background of blue honeysuckle mainly focusses on the morphology, palynology, and chromosomes of this plant. With respect to molecular marker-based research, only RAPD and SSR analysis has previously been reported. The SRAP technique was first proposed by Li & Quiros (2001), and has subsequently been applied in the analysis of the genetic diversity of several commercially important plants, including cucumber (Yeboah *et al.*, 2007), tomato (Mane *et al.*, 2013), and apple (Liu *et al.*, 2016). To date, however, there has been comparatively little work on the application of SRAP analysis in blue honeysuckle characterisation. Our findings in this indicate that SRAP markers can be used to identify the relationships among blue honeysuckle germplasms, and will provide a basis for further studies on the genetic diversity of this plant.

Our cluster analysis of blue honeysuckle based on SRAP marker data revealed that the Changbai Wild and Great Khingan Wild germplasms were assigned to different groups, indicating a distant genetic relationship among these germplasms. Our findings also indicate that cultivar classification has been based on genotype composition. In addition, we established that the polymorphisms of all 58 germplasms can be assessed using SRAP markers, with a polymorphic rate of 83.1%, thereby providing evidence of the broad genetic basis of the assessed blue honeysuckle specimens. In terms of genotypic variation, we observed that germplasms from Russia are characterised by a larger degree of polymorphism than those from China. The large genetic differentiation between these two blue honeysuckle populations can plausibly be attributed to variations in the centres of origin and/or the influence of artificial selection. Given its broad genetic diversity, we would anticipate that blue honeysuckle has considerable potential for variety cultivation and genetic breeding.

Our cluster analysis results revealed that Berel and hybrid L2, with a similarity coefficient of 0.53, are the two most distantly related germplasms, whereas the similarity coefficient of 0.96 obtained for the germplasms Vladivostok No. 24 and No. 28, identifies this pair to be the most closely related. The clustering of unknown genotype No. 23 and Vladivostok germplasms in the same group would tend to indicate that this unknown genotype has probably originated in Vladivostok or is a hybrid of Vladivostok germplasms, which accordingly warrants further research. We found that the 58 germplasms could be grouped into seven groups at a similarity coefficient of 0.74. Huo & Qin (2012) used a RAPD technique to analyse the relationships between six wild blue honeysuckle populations in northeast China, demonstrating a generally close relationship among these populations. The exception in this regard was the Mohe population, which was found to be separated from the other five populations by a somewhat greater genetic distance. The population from Mohe was established to be that of *Lonicera edulis*, whereas those from Changbai and Lesser Khingan comprised *Lonicera boczkarnikowii* Plekh (*L. regeliana*). Zhang *et al*., (2016) used an SSR technique to analyse the relationships among sixteen wild blue honeysuckle populations, the 16 germplasms of which were clustered into three groups. The population from Boli was established to be genetically distant from the other others, whereas the populations from Wangqing and Changbai (both in Jilin) were found to be genetically close. Consistent with the findings reported by Huo *et al*., (2005) and Zhang *et al*., (2016), we found in the present study that blue honeysuckle germplasms from Yichun, Shangzhi, Boli, Great Khingan, and Changbai in northeast China could be classified into four groups, whereas the Mohe population was more distantly related to the others. In addition, we detected regional differences among the wild blue honeysuckle in northeast China, which is taken to be indicative of the abundant genetic variation among the populations of blue honeysuckle in different regions. Moreover, we identified this region as a centre for blue honeysuckle genetic diversity, which is consistent with the assessment of Plekhanova *et al*., (1992), who reported north and northeast Asia to be centres of blue honeysuckle genetic diversity.

Acknowledgements

This research was supported by the National Key R&D Program of China (2022YFD1600500), Academic Backbone Project of Northeast Agricultural University (20XG04), China Agriculture Research System of MOF and MARA (CARS-29-10), the Central Government Supports the Reform and Development Fund Talent Training Project of Local Colleges and Universities and Postdoctoral Fund of Heilongjiang Province (LBH-Z21117). Huixin Gang and Feng Sun contributed equally to this work. The authors would like to thank Shiyanjia Lab (www.shiyanjia.com) for the English editing service.

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(Received for publication 7 January 2022)