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Preliminary study on geographical distribution and evolutionary relationships between cultivated and wild adzuki bean (*Vigna angularis* var. *angularis* and var. *nipponensis*) by AFLP analysis

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Abstract

A set of 146 representative adzuki (*Vigna angularis* var. *angularis* and var. *nipponensis*) germplasm from six Asian countries with a tradition of adzuki bean production, together with an outgroup standard rice bean (*Vigna umbellata*), were analysed by amplified fragment length polymorphism (AFLP) methodology using 12 informative primer pairs. A total of 313 unambiguous polymorphic bands were created. According to the dendrogram, using cluster analysis based on AFLP banding, 143 of the accessions were distinct and revealed enough genetic diversity for identification and classification of accessions within *Vigna angularis*. A neighbour-joining tree was generated using the newly developed Innan's nucleotide diversity estimate from the AFLP data. From analysis, seven distinct evolutionary groups, named 'Chinese cultivated', 'Japanese cultivated', 'Japanese complex-Korean cultivated', 'Chinese wild', 'China Taiwan wild', 'Nepal-Bhutan cultivated' and 'Himalayan wild', were detected. Nucleotide diversity with geographical distribution of each group is discussed, regarding the evolutionary relationships between wild and cultivated adzuki beans. The preliminary results indicated that cultivated adzuki beans have been domesticated from at least four progenitors with at least three geographical origins.

Keywords: adzuki bean; AFLP; evolutionary relationships; genetic diversity; geographical distribution; *Vigna angularis*

Introduction

The adzuki bean (*Vigna angularis*) is one of the ancient traditional East Asian legume crops that originated in Asia (Tomooka *et al*., 2003; Zheng *et al*., 1997). Recently, it was revealed that the adzuki bean has been cultivated traditionally also in Bhutan and Nepal (Tomooka *et al*., 2003). It has been widely regarded that the adzuki bean originated in China, and was introduced to Japan and Korea later. This viewpoint was mainly based on the broader morphological diversity of cultivars and discoveries of wild and weedy adzuki types in the east Himalayan region and in Yunan, Shandong and Hubei provinces and other regions of China (Ding, 1959; Dong, 1982; Hu, 1984; Jiang, 1984; Zheng *et al*., 1997). The wild adzuki bean (*V. angularis* var. *nipponensis*) is considered to be the progenitor of the cultivated adzuki bean, and is distributed from the Himalayan region of

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India, Nepal, Bhutan through the mainland of China, Taiwan island of China, the Korean peninsula to Japan (Tomooka *et al.*, 2000, 2003). Therefore, domestication of the cultivated adzuki bean may have occurred elsewhere and formed several evolutionary centres within these areas, which gives a challenge to the mono-centred theory.

There have been articles on the genetic diversity of adzuki based on randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers, but these never discussed the topic of global origin and evolution of adzuki as only the cultivated plant material was involved (Wang *et al.*, 2002; Yee *et al.*, 1999) or both cultivated and wild materials involved came from narrow geographic origin (Mimura *et al.*, 2000; Xu *et al.*, 2000; Yoon *et al.*, 2000). In the present study, a set of representative wild and cultivated azuki bean materials from Indian Himalaya, Nepal, Bhutan, China, Taiwan, Korea and Japan, were collected and analysed at the DNA level using AFLP methodology. The AFLP methodology was chosen to analyse intraspecific diversity in *Vigna angularis* because it had been previously shown to detect polymorphism clearly in these species (Xu *et al.*, 2000; Yee *et al.*, 1999) and Innan's new methods are available to estimate genetic distance from AFLP banding pattern data (Innan *et al.*, 1999). This allows the relationships among cultivated and wild adzuki beans and their natural geographical distributions to be explored, and may help to find the answer for the origin and evolution of cultivated adzuki bean.

Materials and methods

Materials

A total of 146 accessions of adzuki bean with a standard rice bean (*V. umbellata*) CK line-M00, were obtained from the germplasm collections of the Chinese Academy of Agricultural Sciences and the National Institute of Agrobiological Sciences of Japan. Of these, 84 accessions (79 cultivated and five wild) were from China, 36 accessions (24 cultivated, five weedy and seven wild) were from Japan, nine cultivated accessions were from Korea, 12 accessions (nine cultivated and three wild) were from Nepal, three accessions (two cultivated and one wild) were from Bhutan, one wild accession was from Indian and one wild accession was from Taiwan island of China. M00 was from Japan. These accessions are considered to represent the natural distribution of wild adzuki and cultivated adzuki beans in traditional cultivation areas, as well as the genetic diversity of the adzuki bean.

DNA extraction

Seeds of each accession were planted in pots on 8 October 2001 in the greenhouse at temperatures of 25–30°C. Fourteen to 25 days after germination, immature leaves were harvested from five seedlings of each accession. Following lyophilization, frozen immature leaves from individuals of each accession were finely ground with a mortar and pestle in liquid nitrogen. Total genomic DNA was extracted from 200–300 mg of bulked immature leaves of each accession according to the procedure described previously by Dellaporta *et al.* (1983) with minor modification. DNA concentrations were estimated and standardized to 50 ng/μl against known concentrations of DNA on 1% agarose gels stained by ethidium bromide (EtBr). Prepared DNA samples were stored in a refrigerator at −20°C.

AFLP analysis

AFLP fingerprinting was carried out as described by the *AFLP Protocol for Public Release*, Version 2.1 of Key Gene (1994) with minor modifications.

Total genomic DNA digestion and ligation

First, about 0.25 μg of genomic DNA were incubated for 3 h at 37°C in 20 μl of solution containing 2.5 U *Eco*RI, 2.5 U *Mse*I, 10 mmol Tris hydrogen acetate of pH 7.5, 10 mmol magnesium acetate, 50 mmol potassium acetate, 5 mmol dithiothreitol (DTT) and 50 ng/μl bovine serum albumin (BSA). Then, 10 μl of solution containing 5 pmol *Eco*RI adapters, 5 pmol *Mse*I adapters (Table 1), 1 U T4DNA-ligase, 1 mmol ATP, 10 mmol Tris acetate of pH 7.5, 10 mmol magnesium acetate, 50 mmol potassium acetate, 5 mmol DTT and 50 ng/μl BSA was added, and the incubation was continued for one night at 37°C. After ligation, the reaction mixture was heat-inactivated at 65°C for 10 min and diluted to 100 μl with 10 mmol Tris-HCl and 0.1 mmol EDTA of pH 8.0, for storage in a refrigerator at −20°C.

PCR reaction

Two pre-selective primers and 12 pairs of selective primers were synthesized to be complementary to the adapter/restriction site sequences and to carry selective 3′ nucleotides. *Eco*RI selective primers included 12 *Eco*RI + 3 primer, and *Mse*I selective primers included eight *Mse*I + 3 primer, with a total of l2 *Eco*RI/*Mse*I + 3 primer combinations. Adapter, + 0 and 3 primer sequences are presented in Table 1.

A 20 μl pre-selective PCR mixture contained 5 μl of the restriction-ligation DNA product (after 5-fold dilution), 0.6 μl of 50 ng/μl E00 primer and 0.6 μl of 50 ng/μl M00

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Table 1. The sequences of adapters and primers

Adapter and primers	Nucleotide sequences	Application
*Eco*RI adapter	5′-CTC GTA GAC TGC GTA CC-3′ 3′-CTG ACG CAT GGTTAA-5′	Adapter ligation
*Mse*I adapter	5′-GAC GAT GAG TCC TGA G-3′ 3′-TA CTC AGG ACT CAT-5′	Adapter ligation
*Eco*RI primer		
E00	5′-GACTGCGTACCAATTC-3′	Pre-selective amplification
E32	5′-GACTGCGTACCAATTC AAC-3′	Selective amplification
E33	5′-GACTGCGTACCAATTC AAG-3′	
E38	5′-GACTGCGTACCAATTC ACT-3′	
E39	5′-GACTGCGTACCAATTC AGA-3′	
E44	5′-GACTGCGTACCAATTC ATC-3′	
E45	5′-GACTGCGTACCAATTC ATG-3′	
E47	5′-GACTGCGTACCAATTC CAA-3′	
E50	5′-GACTGCGTACCAATTC CAT-3′	
E59	5′-GACTGCGTACCAATTC CTA-3′	
E75	5′-GACTGCGTACCAATTC GTA-3′	
E83	5′-GACTGCGTACCAATTC TCA-3′	
E93	5′-GACTGCGTACCAATTC TTG-3′	
*Mse*I primer		
M00	5′-GATGAGTCCTGAGTAA-3′	Pre-selective amplification
M32	5′-GATGAGTCCTGAGTAA AAC-3′	Selective amplification
M33	5′-GATGAGTCCTGAGTAA AAG-3′	
M38	5′-GATGAGTCCTGAGTAA ACT-3′	
M39	5′-GATGAGTCCTGAGTAA AGA-3′	
M45	5′-GATGAGTCCTGAGTAA ATG-3′	
M47	5′-GATGAGTCCTGAGTAA CAA-3′	
M50	5′-GATGAGTCCTGAGTAA CAT-3′	
M75	5′-GATGAGTCCTGAGTAA GTA-3′	

primer, 1.6 μl of dNTP mixture (2.5 mM each), 0.08 μl of 5 U/μl *Taq* polymerase and 2 μl of 10 × PCR buffer (containing 15 mmol $MgCl_2$). Samples were subjected to 25 thermal cycles of 94°C denaturation for 30 s, 56°C annealing for 1 min and 72°C extension for 1 min using a GeneAmp PCR system 9700 (Applied Biosystems, USA). After thermal cycling, amplified products were diluted 20-fold with 10 mmol Tris-HCl and 0.1 mmol EDTA of pH 8.0 for selective PCR, and storage in a refrigerator at −20°C.

A 20 μl selective PCR mixture contained 5 μl of a diluted solution of pre-selective PCR products, 0.6 μl of 50 ng/μl *Eco*RI + 3 primer, 0.6 μl of 50 ng/μl *Mse*I + 3 primer, 1.6 μl dNTP (2.5 mmol each), 0.08 μl of 5 U/μl *Taq* polymerase and 2.0 μl of 10 × PCR buffer. The PCR was programmed to a touchdown cycle profile as follows: 94°C, 30 s; 65°C (−0.7°C per cycle), 30 s; 72°C, 60 s, during 13 cycles; followed by 23 more cycles at 94°C, 30 s; 56°C, 30 s; 72°C, 60 s. The selective PCR productions were stored in a refrigerator at −20°C.

Electrophoresis

After selective PCR, 2 μl of each amplified product as well as 10 and 50 bp DNA step ladder markers (Promega, USA) mixed with 2 μl of denaturation buffer (98% formamide, 10 mmol EDTA, 0.10% each of bromophenol blue and xylene cyanol FF) were denatured for 3–4 min at 95°C and placed immediately on ice. Two microlitres of each denatured product were electrophoresed in 6% (w/v) acrylamide monomer gels (containing 7.5 mol urea) in 0.5 TBE, at 70 W constant power in a EC160 (E-C Apparatus Co., USA) for 1 h and 40 min. After electrophoresis, separated AFLP products were visualized in the gels by silver staining as described in the Promega DNA Silver Staining System (Promega).

Data processing methodology

AFLP bands were scored as present '1' and absent '0', and only bands showing unambiguous amplification were entered into a data matrix. Genetic similarity was calculated with Jaccard's coefficient (1908). Jaccard's coefficient = $a/(n - d)$, where n is total number of polymorphic bands, a is number of bands present in both samples and d is the number of bands absent in both samples. Jaccard's similarity coefficients (JSCs) range from 0 (all bands between accessions were different) to 1 (all bands between accessions were identical). SAHN cluster analysis is conducted using the UPGMA

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(unweighted pair-group method with an arithmetic average) methodology of Rohlf (2000).

Nucleotide diversity, defined as the average number of pairwise nucleotide changes per site, was estimated based on the recently designed method using AFLP polymorphism (Innan *et al.*, 1999). In using this method we assume that *Vigna angularis* is basically inbreeding. A neighbour-joining tree was constructed based on nucleotide diversity using PHYLIP version 3.57 (Felsenstein, 1992). Bootstrap analysis was performed with 1000 replications.

Results

AFLP polymorphism of adzuki genetic resources

Analysis of the 146 adzuki accessions with 12 informative AFLP primer pairs generated 580 unambiguous fragments, 313 (53.93%) of which were polymorphic (Fig. 1). The UPGMA dendrogram (Fig. 2) based on Jaccard's similarity coefficient showed the biggest difference of about 15% among adzuki entries, but 143 of them can be identified out of the 146 entry accessions.

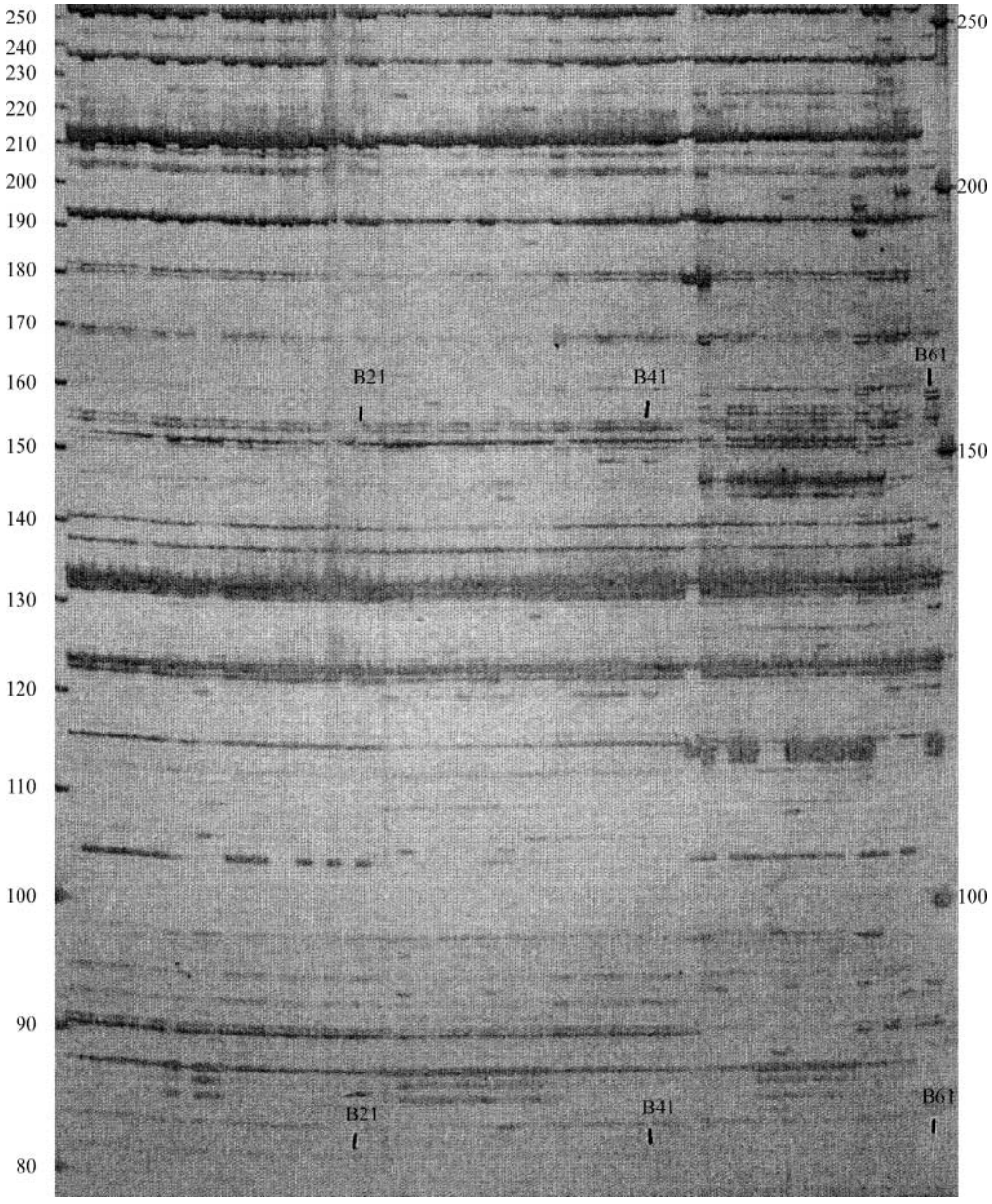

Fig. 1. AFLP banding patterns produced by primer pair E75/M75.

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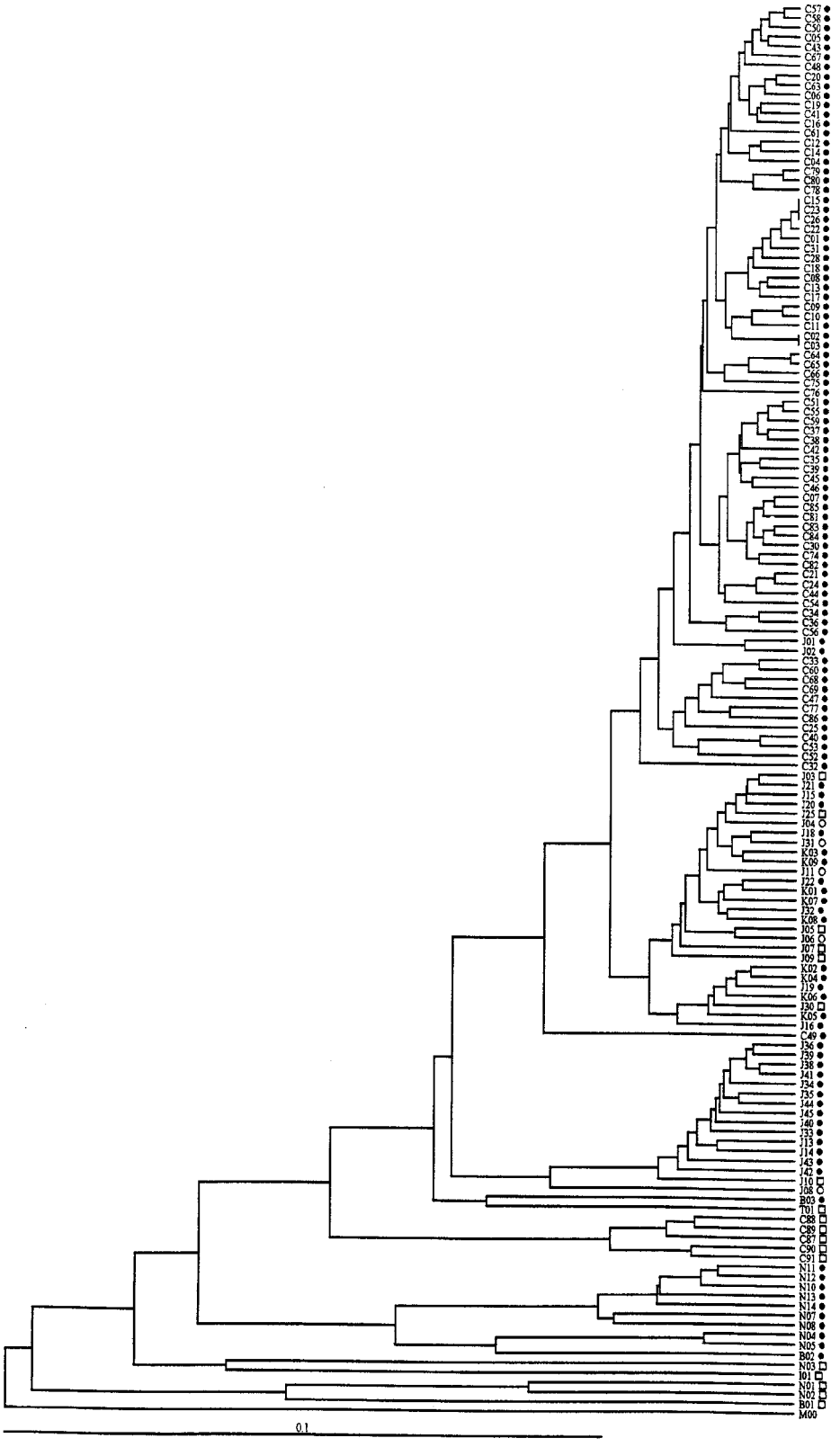

Fig. 2. Dendogram of UPGMA cluster analysis based on Jaccard's similarity matrix converted from AFLP polymorphism data.

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AFLP fingerprinting revealed a high identification rate that can be used for classification within adzuki species, and for exploration of relationships between its evolutionary and geographical distributions.

Evolutionary groups within adzuki species

The neighbour-joining tree based on the matrix of Innan's inferred nucleotide diversity presented in Fig. 3 gives topographical identification of seven distinct evolutionary groups that are highly correlated to the geographic origin. The seven evolutionary groups indicate a clear pattern of geographical differentiation and therefore are named as 'Chinese cultivated', 'Japanese cultivated', 'Japanese complex-Korean cultivated', 'China Taiwan wild', 'Chinese wild', 'Nepal-Bhutan cultivated' and 'Himalayan wild' (Table 2). The out-group, standard CK rice bean (*V. umbellata*) accession M00, was separated from all the seven groups with a long genetic distance to each of them, which proved the reliability of Innan's methodology. This result clearly indicated that the revealed genetic diversity showed geographic cline. However, morphological evaluation of seed colours of these accessions provided no explanation for these evolutionary groups, even if the seed colour has been thought the most representative character for genetic diversity and evolutionary levels traditionally, although all the seeds of wild and weedy accessions are black and black-marble in this study (Table 2).

Evolutionary group 'Chinese cultivated' includes 80 accessions of var. *angularis*, 78 from China and two from Japan; evolutionary group 'Japanese cultivated' contains 15 Japanese cultivars, one Chinese cultivar and one Japanese wild accession; evolutionary group 'Japanese complex-Korea cultivated' consists of 27 accessions (which include var. *angularis*, var. *nipponensis* and weedy forms) from Japan and nine Korean cultivated azuki beans; evolutionary group 'China Taiwan wild' contains one Taiwan wild and one Bhutan cultivated accession; evolutionary group 'Chinese wild' includes only five wild (var. *nipponensis*) accessions from China; evolutionary group 'Nepal-Bhutan cultivated' consists of nine Nepal cultivated accessions and one Bhutan cultivated accession; evolutionary group 'Himalayan wild' contains all the five wild accessions from the Himalayan region of Bhutan (one), Nepal (three) and India (one) (see Table 2).

Evolutionary relationships within and among evolutionary groups

Judging from the neighbour-joining tree based on Innan's nucleotide diversity (Fig. 3), the genetic distances

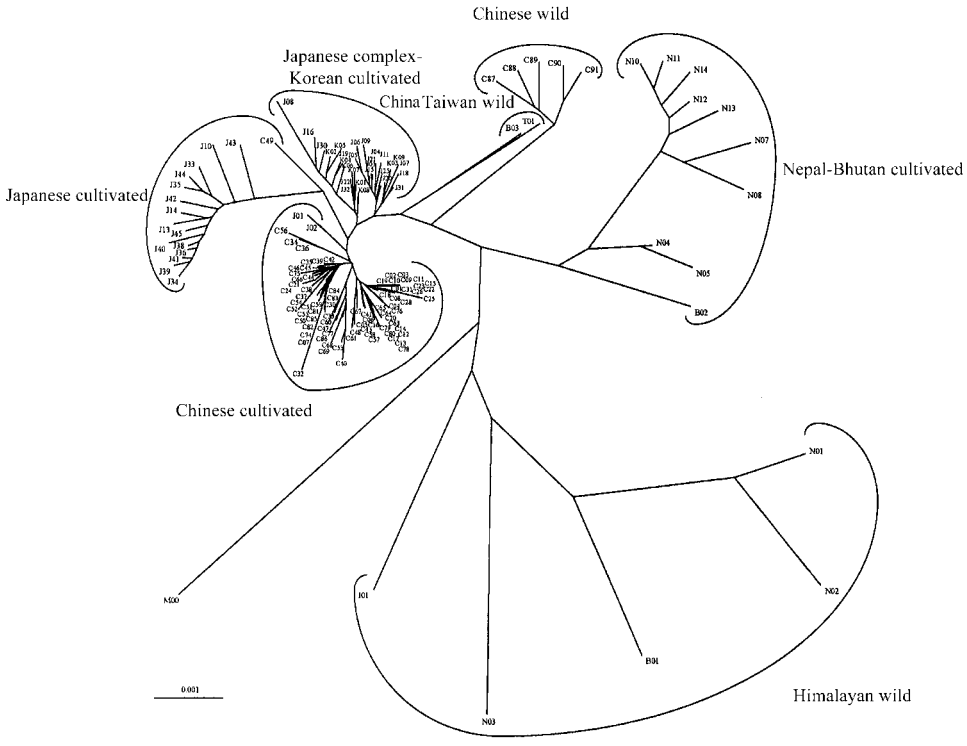

Fig. 3. A neighbour-joining tree based on pairwise distance using Innan's nucleotide diversity data (showing evolutionary groups).

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Table 2. Seven evolutionary groups of *Vigna angularis* and their distribution on seed colours

Evolutionary group	Accessions	Distribution of seed colours
Chinese cultivated	C01, C02, C03, C04, C05, C06, C07, C08, C09, C10, C11, C12, C13, C14, C15, C16, C17, C18, C19, C20, C21, C22, C23, C24, C25, C26, C28, C30, C31, C32, C33, C34, C35, C36, C37, C38, C39, C40, C41, C42, C43, C44, C45, C46, C47, C48, C50, C51, C52, C53, C54, C55, C56, C57, C58, C59, C60, C61, C63, C64, C65, C66, C67, C68, C69, C74, C75, C76, C77, C78, C79, C80, C81, C82, C83, C84, C85, C86, J01, J02	56 red, 9 white, 2 yellow, 2 green, 6 black-marble, 5 red stripe
Japanese cultivated	C49, J10[a], J13, J14, J33, J34, J35, J36, J38, J39, J40, J41, J42, J43, J44, J45	9 red, 1 white, 3 black, 3 black-marble
Japanese complex-Korean cultivated	J03[a], J04[b], J05[a], J06[b], J07[a], J08[b], J09[a], J11[b], J15, J16, J18, J19, J20, J21, J22, J25[a], J30[a], J31[b], J32, K01, K02, K03, K04, K05, K06, K07, K08, K09	13 red, 2 white, 1 green, 12 black-marble
Chinese Taiwan wild	T01[a], B03	1 black-marble, 1 red/black-marble
Chinese wild	C87[a], C88[a], C89[a], C90[a], C91[a]	5 black-marble
Nepal-Bhutan cultivated	B02, N04, N05, N07, N08, N10, N11, N12, N13, N14	9 red, 1 brown
Himalayan wild	B01[a], I01[a], N01[a], N02[a], N03[a]	5 black marble

Accessions from: C, China; B, Bhutan; J, Japan; K, Korea; N, Nepal; I, India; T, Taiwan island of China.
[a]wild accession; [b]weedy accession.

between evolutionary groups of 'Chinese cultivated', 'Japanese cultivated' and 'Japanese complex-Korea cultivated' were small. Some pervasions between each other were obvious. In the evolutionary group 'Chinese cultivated', two Japanese cultivars were included. In the evolutionary group 'Japanese cultivated', one Chinese cultivar and one Japanese wild accession were included. In the evolutionary group 'Japanese complex-Korea cultivated' all the other accessions of cultivated, weedy and wild within Japan and Korea were included. It showed close consanguinity among the three evolutionary groups, and predicted that they may have similar ancestors. Even so, of 79 Chinese cultivated accessions, 78 are included in the evolutionary group 'Chinese cultivated', only one was out of this group and two Japanese cultivated accessions were in this group, indicating a unique evolutionary group to other two closely related groups. The majority of Japanese cultivars were divided into two evolutionary groups of 'Japanese cultivated' and 'Japanese complex-Korea cultivated'. One of the Japanese wild accessions J10 was classified into evolutionary group 'Japanese cultivated'. The other six Japanese wild and five Japanese weedy accessions were classified into evolutionary group 'Japanese complex-Korea cultivated'. The results of the above show the clear distinction between the genetic structures of the two evolutionary groups 'Japanese cultivated' and 'Japanese complex-Korea cultivated', and the wild accession J10 should be the similar ancestor type of the evolutionary group 'Japanese cultivated'. The evolutionary group 'Japanese complex-Korea cultivated' included all the other Japanese accessions of cultivated, weedy and wild, and all the nine cultivated Korean accessions, indicating extremely close relationships among the accessions within this group, and may reveal direct evolutionary linkages between cultivated adzuki from Japan/Korea and weedy/wild adzuki from Japan, or predict the genetically similar ancestor of this evolutionary group.

The genetic distance among wild evolutionary groups 'China Taiwan wild', 'Chinese wild' and the evolutionary group 'Japanese complex-Korea cultivated' were much shorter than that to 'Himalayan wild' in the neighbour-joining tree. It indicated closer consanguinity among the three evolutionary groups, and more distant consanguinity from 'Himalayan wild' to the three evolutionary groups. Within the evolutionary group 'Nepal-Bhutan cultivated', all the 10 accessions were cultivated, but all were well differentiated each other, showing greater genetic diversity within this group of cultivars. This group itself was unique and had a extremely large genetic distance to other cultivated groups, which provides an important gene pool for genetic improvement and breeding for the adzuki bean in the future. The evolutionary group 'Himalayan wild' contained all five wild accessions from the Himalayan region of Bhutan, Nepal and Indian, showing the greatest differentiation within group, indicating the background of the wild gene pool with great genetic diversity. This group was unique and very far from the other groups by genetic distance. 'Nepal-Bhutan cultivated' group and 'Himalayan wild'

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group both originated in the Himalayan region which is well isolated from any other adzuki distribution areas, which predicts that there should be some kind of evolutionary relationship between them, although they were genetically very far from each other.

Centres of origin for the cultivated adzuki bean

According to the preliminary analysis based on the experimental results, there should be several centres of origin and evolution for the cultivated adzuki bean in Asia. The cultivated adzuki bean of China should have been domesticated within China, and share the same type of wild progenitors. The cultivated adzuki bean of Japan belongs to two genetic types that should have originated from two types of wild progenitor in the archipelago of Japan. The Korean cultivated adzuki bean, accorded with one of the two genetic types of Japanese cultivated, should have been domesticated from a common type of wild progenitor. Indigenous cultivated adzuki bean from the west side of the Himalayan mountains should have different kinds of progenitor in this area. So, genetic types of cultivated adzuki bean should have been domesticated from at least four progenitors with at least three geographical origins.

Discussion

Traditional studies on the origin of the cultivated adzuki bean have been based mainly on morphological classification, rate of successful hybridization and isozyme analysis. There has been no tangible answer generated from traditional studies on whether the cultivated adzuki bean shared only one common progenitor in the same geographical origin, although the conclusion of the previous research was that the cultivated adzuki bean had been domesticated from a wild progenitor. Based on DNA nucleotide diversity revealed by AFLP, this preliminary study affirmed for the first time that the cultivated adzuki bean originated from several evolutionary centres of origin, which paved a path for further exploration of such studies. But, this preliminary study gave no answers to what kind of wild types should be the progenitors for indigenous cultivated adzuki bean from China and from the Nepal-Bhutan district and what evolutionary relationships are among the indigenous cultivars from China, Japan and Korea. The shortcomings mentioned above in this study obviously come from the less than sufficient coverage of the genetic resources of adzuki bean (wild and weedy). To overcome the shortcomings in future research, a thorough collection of cultivated, wild and weedy entries from China, the Korean peninsula and the Himalayan areas should be involved.

Another shortcoming in this study is the lack of adzuki cultivar entries from the south and south-west parts of China, which formed a gap in the adzuki distributive band between East and South Asia. This shortcoming may have blocked the discovery of the linkage of evolutionary relationships of origin in the study, and set another example to prove the importance of sampling in research. The sampling gap must be filled in future research in order to achieve more systematic data on genetic diversity.

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