Identification of Rabbiteye Blueberry Cultivars (*Vaccinium ashei* Reade) and Analysis of Genetic Relationships Using Amplified Fragment Length Polymorphism (AFLP)

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Summary: Proper cultivar identification is a requisite for commercial planting and breeding nurseries of cross-pollinated blueberry (*Vaccinium ashei* Reade) cultivars to insure high crop yields and optimize germplasm maintenance and utilization. Fourteen rabbiteye blueberry cultivars and three non-identified clones were screened with amplified fragment length polymorphism (AFLP) analysis with the aim of developing a fast and reliable identification technique. The selective primer pair applied (M-CTG/ E-ACC), which was previously tested, resulted in a large number of reproducible polymorphic fragments for cultivar identification. After comparison of the AFLP fingerprints, the Jaccard similarity indexes were calculated, and an UPGMA dendrogram was constructed. It was revealed that the three non-identified clones belong to the *'Tifblue'* cultivar. Moreover, AFLP technique proved to be a fast, successful and reliable way in rabbiteye blueberry identification.

Key words: AFLP, cultivar identification, genetic relationship, rabbiteye blueberry

Introduction

In general, cultivars of rabbiteye blueberry (*Vaccinium ashei* Reade), which is a cross-pollinated species, are identified by morphological characteristics. Despite the fact that blueberry cultivars are clonally propagated, and thus assumed to be genetically identical, some degree of morphological variation due to plant age or growing conditions might occur *(Aruna* et al., 1995). It is not unusual for commercial growers and breeders to encounter difficulties in accurately identifying plants whose clonal origin is questionable. Proper cultivar identification is necessary in commercial rabbiteye blueberry planting and breeding nurseries to insure high quality and quantity crop yields and to optimize germplasm maintenance and utilization. Several studies have reported that RAPD (randomly amplified polymorphic DNA) techniques are acceptable for identification of blueberry cultivars *(Aruna* et al., 1995; *Qu & Hancock*, 1997; *Levi & Rowland*, 1997, *Arce-Johnson* et al., 2001, *Burgher* et al., 2002). After the introduction of a new technique, AFLP (amplified fragment length polymorphism), which does not require nucleotide sequence information and allows the specific co-amplification of high numbers of restriction fragments *(Vos* et al., 1995). This new method has been successfully applied for molecular genetic analyses of peaches *(Shimada* et al., 1998), genetic characterization of Asian chestnut varieties *(Yamamoto* et al., 1998), diversity detection of hop cultivars *(Hartl & Seefelder*, 1998, *Townsend* et al. 2000), analyses of the genetic relationship among Japanese and Chinese persimmon cultivars *(Kanazaki* et al., 2000), detection of the clonal structure of a dwarf bamboo population *(Suyama* et al., 2000), and identification of genotypes in apple rootstock *(Zhu* et al. 2001). Comparative analysis of AFLP and RAPD *Polashock & Vorsa* (1997); agreed *Grzebelus* et al. (2001) that although AFLP analyses require more steps and thus are consequently more costly, the units of information (i.e. number of polymorphic bands) from a single AFLP reaction are greater than those from a typical RAPD reaction. Subsequently, this reduces the relative cost of AFLP and increases the speed and accuracy of identification. The objective of this study was to apply the AFLP technique for identification of rabbiteye blueberry cultivars with unknown origin.

Material and method

Plant material

Fourteen known rabbiteye blueberry (*Vaccinium ashei* Reade) cultivars: *'Bluebell' (Bb), 'Bluegem' (Bg), 'Briteblue' (Br), 'Callaway' (Ca), 'Climax' (Cl), 'Coastal' (Co), 'Delite' (D), 'Festival' (F), 'Gardenblue' (Gb), 'Homebelle' (Hb), 'Nobilis' (N), 'Southland' (S), 'Tifblue'*

(T), and 'Woodard' (W), and three clones of an unknown cultivar (*U1, U2,* and *U3*) were analysed in this study. The three unknown cultivars were suspected to belong to the same cultivar because of their similar morphological and phenological characteristics, for example, the shapes of their leaves, and similar flowering and ripening times. The pedigrees of the examined cultivars and their parents are shown in *Table 1*. All the cultivars and the unknown clones were grown in the blueberry plantation of the Field Science Center, Tohoku University, Nango.

Table 1 The pedigree of rabbiteye blueberry cultivars used in the study

Sample name	Cultivars	Pedigree[a]	Source
Bb	Bluebell*	Callaway X Ethel	Oozeki corp.[b]
Bg	Bluegem*	Open pollinated selection of Tifton31 X Callaway	Oozeki corp.
Br	Briteblue*	Ethel X Callaway	Oozeki corp.
Ca	Callaway*	Myers X Black Giant	Oozeki corp.
Cl	Climax*	Callaway X Ethel	Oozeki corp.
Co	Coastal*	Myers X Black Giant	Oozeki corp.
D	Delite*	T-14 X T-15 (Georgia selections)	Oozeki corp.
F	Festival (T-172)*	Tifblue X T- 65	Oozeki corp.
Gb	Gardenblue*	Myers X Clara	Oozeki corp.
Hb	Hombelle*	Myers X Black Giant	Oozeki corp.
N	Nobilis (T-100)*	Tifblue X Mendito	Oozeki corp.
S	Southland*	Gardenblue X Ethel	Oozeki corp.
T	Tifblue*	Ethel X Clara	Oozeki corp.
U1	Unknown 1*	?	unknown
U2	Unknown 2*	?	unknown
U3	Unknown 3*	?	unknown
W	Woodard*	Ethel X Callaway	Oozeki corp.

[a] Corvallis Vaccinium catalog, 2004

[b] Oozeki Nursery Corporation, Imaizumi, Tsuchiura, Ibaraki 300–0001, Japan.

* the 14 cultivars and unknown cultivars used in this study

DNA extraction

Young leaves were collected from the field-grown plants, and were stored at –20 °C. DNA extraction was carried out following the protocols described in the DNeasy® Plant Mini Kit handbook. DNA extract concentrations were measured using a Gene Quant RNA/DNA calculator.

DNA amplification

Restriction digestion and ligation of the adaptors were carried out using the AFLP Core Reagent Kit according to the slightly modified protocol of Life Technologies. Pre-selective and selective amplification were performed with the AFLP Plant Mapping Kit according to the Perkin-Elmer's protocol. In the selective amplification step, one pair of *Mse*I (M-) and *Eco*RI (E-) primers (M-CTG/ E-ACC), which was shown to be the most informative and reliable during a preliminary investigation (data not shown), was used. The polymerase chain reaction was run according to the protocol of GeneAmp PCR System 9700 (Perkin-Elmer). AFLP samples were analyzed using an ABI PRISM 310 Genetic Analyzer and GeneScan analysis software (Perkin-Elmer), and compared according to the presence or absence and intensity of the fragments.

Data analysis

The presence or absence of fragments was evaluated in the reproducible range of fragment sizes (60 to 400 bp) for the selected primer pair. Similarities in the AFLP fingerprints between pairs of cultivars were calculated using Jaccard index ($a/(a+b+c)$), where a is the number of shared bands, and b and c are the number of bands present in one sample but absent in the other, respectively. An UPGMA (Unweighted Pair-Group Method using Arithmetic averages) dendrogram was then constructed, based on the Jaccard similarities matrix using the PHYLIP 3.57c (Phylogeny Inference Package) software (Felsenstein, 1995).

Results and discussion

The AFLP technique was found to be effective in identifying the unknown rabbiteye blueberry cultivars, which revealed that 'Unknown 1, 2 and 3' clones had the same fingerprints as the '*Tifblue*' cultivar *(Figure 1)*. The selective primer pair (M-CTG/ E-ACC) proved to be reliable, and produced a large number of polymorphic fragments *(Table 2)*.

The lengths of the evaluated fragments ranged between 60 and 400 base pairs. The number of fragments per sample was the highest in the '*Coastal*' cultivar (53) and the lowest in the '*Callaway*' and '*Climax*' cultivars (42 each). The number of fragments per sample pairs was found to be the highest between the '*Coastal*'–'*Tifblue*' and '*Coastal*'–'*Unknown 1–3*' pairs (68), and lowest between the '*Callaway*'–'*Climax*' pair (48). The number of polymorphic fragments was highest between the 'Coastal'?'*Tifblue*' and the '*Coastal*'–'*U1–3*' pairs (33), and lowest between the '*Unknown 1*–3' clones and

Table 2 Amplified fragment length polymorphisms (AFLPs) detected with M-CTG/E-ACC selective primer pair in 14 cultivars of rabbiteye blueberry

Primer pair	Sizevange of fragments (bp)	Mean of fragments per sample	Mean of polymorphic fragments per pair of samples	Mean polymorpisms between pairs of cultivars (%)
M-CTG/ E-ACC	60 – 400	47.9 (42–53)	21.5 (0–33)	36.5 (0–48.5)

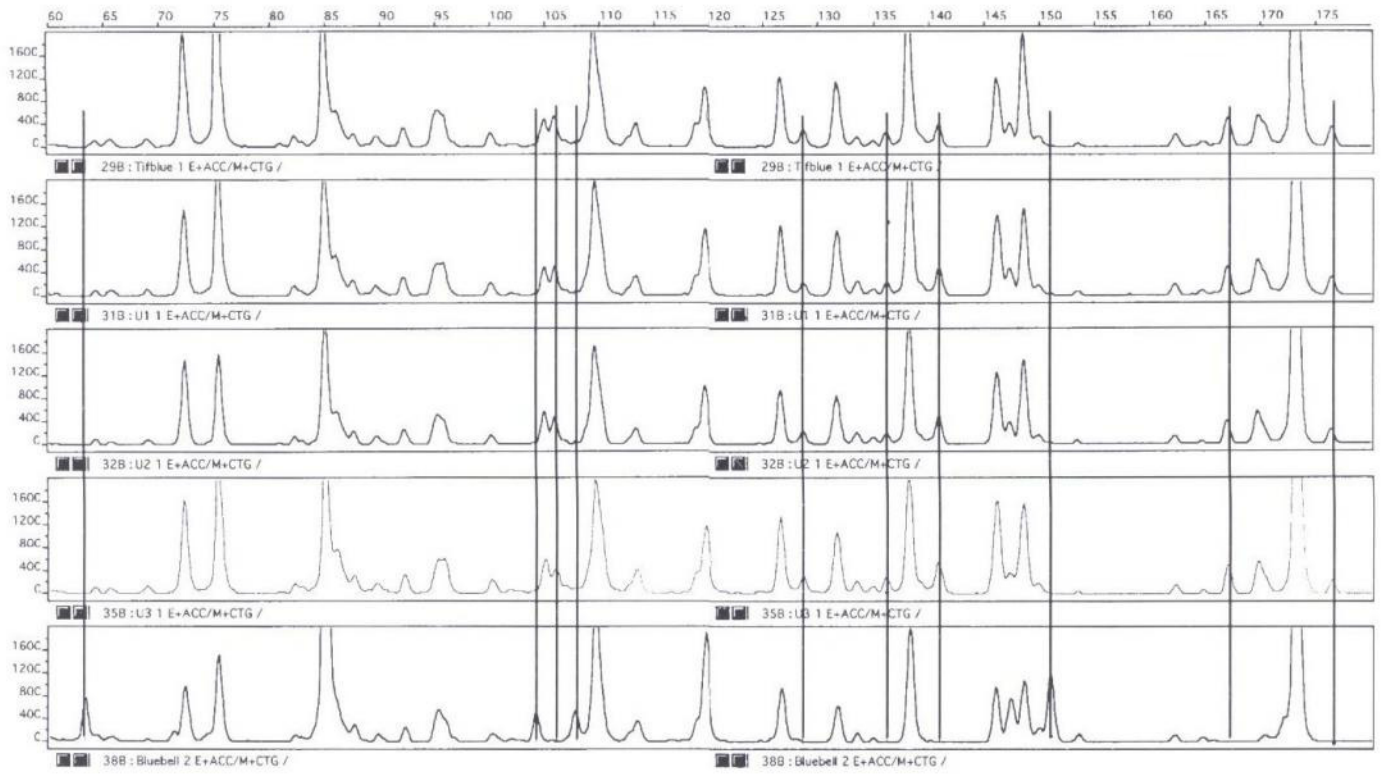

Figure 1 Samples of AFLP fingerprints (ABI PRISM 310) of '*Tifblue*', '*Unknown 1, 2* and *3*'and '*Bluebell*' cultivars

Table 3 Jaccard Similarity indexes between each pair of rabbiteye blueberry cultivars

Cultivars	Bb	Bg	Br	Ca	Cl	Co	D	F	Gb	Hb	N	S	T	U1	U2	U3	W
Bluebell	1																
Bluegem	0.633	1															
Blueray	0.661	0.714	1														
Callaway	0.586	0.698	0.8	1													
Climax	0.643	0.636	0.731	0.75	1												
Coastal	0.635	0.554	0.603	0.638	0.532	1											
Delite	0.868	0.672	0.702	0.655	0.685	0.619	1										
Festival	0.629	0.547	0.597	0.55	0.525	0.552	0.613	1									
Gardenblue	0.621	0.614	0.643	0.686	0.564	0.672	0.691	0.583	1								
Homebelle	0.712	0.597	0.65	0.722	0.576	0.793	0.724	0.594	0.759	1							
Nobilis	0.649	0.559	0.614	0.593	0.564	0.59	0.661	0.638	0.6	0.638	1						
Southland	0.714	0.679	0.709	0.6	0.63	0.547	0.759	0.617	0.636	0.59	0.552	1					
Tifblue	0.696	0.661	0.661	0.559	0.614	0.515	0.678	0.683	0.593	0.554	0.741	0.714	1				
Unknown1	0.639	0.661	0.661	0.559	0.614	0.515	0.678	0.683	0.593	0.554	0.741	0.714	1	1			
Unknown2	0.639	0.661	0.661	0.559	0.614	0.515	0.678	0.683	0.593	0.554	0.741	0.714	1	1	1		
Unknown3	0.639	0.661	0.661	0.559	0.614	0.515	0.678	0.683	0.593	0.554	0.741	0.714	1	1	1	1	
Woodard	0.702	0.667	0.69	0.542	0.632	0.563	0.714	0.607	0.569	0.607	0.542	0.755	0.702	0.702	0.702	0.702	1

between the '*Tifblue*' and '*Unknown 1–3*' samples (0), indicating that there were no differences between the three unknown clones or between the '*Unknown 1*–3' clones and the '*Tifblue*' cultivar. These data were also reflected in the ratio of polymorphisms between the pairs.

The Jaccard similarity results *(Table 3)* revealed complete similarities (value 1) between the '*Tifblue*' cultivar and '*Unknown 1–3*'. They also showed that all the cultivars are closely related, even the least related cultivars ('*Coastal*'?'*Tifblue*' and '*Coastal*'–'*Unknown 1–3*') had value higher than 0.5. The UPGMA dendrogram (Fig. II-2) constructed based on the similarity indices agreed with the pedigree data of the cultivars as follows: the '*Ethel*' cultivar was a common ancestor of the '*Bluebell*', '*Delite*', '*Southland*' and '*Woodard*' cultivars; the '*Bluegem*', '*Briteblue*', '*Callaway*' and '*Climax*' cluster was linked by the '*Callaway* 'cultivar; the '*Tifblue*' and the '*Unknown 1–3*' clones seemed to be the same cultivar sharing cluster with the '*Nobilis*' and '*Festival*' cultivars because of their '*Tifblue*' mother; and the last cluster composed of the siblings of the '*Coastal*', '*Homebelle*' and '*Gardenblue*' cultivars derived from the 'Myers' cultivar seemed more separate than the previous three clusters.

The results of this study show that the AFLP technique is a reliable method for identifying rabbiteye blueberry cultivars and detecting the genetic relationships between cultivars, even if the genetic distances were close.

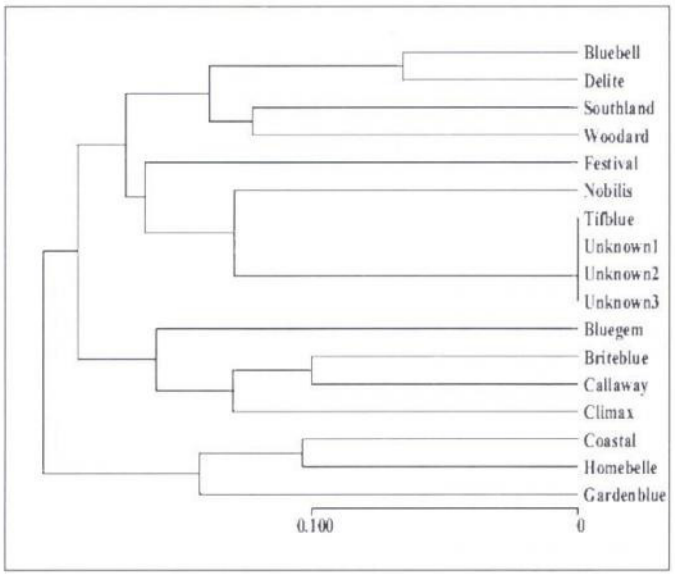

Figure 2 UPGMA dendrogram based on AFLP fragment patterns of fourteen rabbiteye blueberry cultivars and the three unknown clones

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