A rapid and reliable PCR-restriction fragment length polymorphism (RFLP) marker for the identification of *Amaranthus cruentus* species

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A rapid and reliable PCR-restriction fragment length polymorphism (RFLP) marker was developed to identify the *Amaranthus cruentus* species by comparing sequences of the starch branching enzyme (*SBE*) locus among the three cultivated grain amaranths. We determined the partial *SBE* genomic sequence in 72 accessions collected from diverse locations around the world by direct sequence analysis. Then, we aligned the gene sequences and searched for restriction enzyme cleavage sites specific to each species for use in the PCR-RFLP analysis. The result indicated that *MseI* would recognize the sequence 5′-T/TAA-3′ in intron 11 from *A. cruentus SBE*. A restriction analysis of the amplified 278-bp portion of the *SBE* gene using the *MseI* restriction enzyme resulted in species-specific RFLP patterns among *A. cruentus*, *Amaranthus caudatus* and *Amaranthus hypochondriacus*. Two different bands, 174-bp and 104-bp, were generated in *A. cruentus*, while *A. caudatus* and *A. hypochondriacus* remained undigested (278-bp). Thus, we propose that the PCR-RFLP analysis of the amaranth *SBE* gene provides a sensitive, rapid, simple and useful technique for identifying the *A. cruentus* species among the cultivated grain amaranths.

**Key Words:** PCR-RFLP, *Amaranthus cruentus*, species identification, *SBE* gene.
Materials and Methods

Plant materials

A total of 72 accessions from three species of grain amaranths were used (Table 1). All accessions were obtained from collections at the United States Department of Agriculture (USDA), USA and Shinshu University, Japan. The samples in these collections originated in the Americas (Argentina, Bolivia, Brazil, Chile, Guatemala, Mexico, Peru, Puerto Rico and the United States), Africa (Ghana, Nigeria, Uganda, Zaire and Zambia) and Asia (Afghanistan, Bhutan, China, India, Nepal, Pakistan and Sri Lanka).

Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from young leaves using the CTAB method (Murray and Thompson 1980) or the DNeasy Plant Mini kit (Qiagen, Hilden, Germany). The quality and concentration of the DNA were evaluated by viewing samples in agarose gels and by a ND-1000 Nanodrop spectrophotometer (Nanodrop Technologies). A fragment of the SBE genomic DNA was amplified by using gene-specific primers, which have been designed previously (Table 2). PCR reactions were conducted in 50 μl volumes containing 2 μl of total DNA, 5 μl of 10× PCR buffer, 4 μl of 2.5 mM dNTP mixture, 10 pmol of each primers and 0.5 μl of EX Taq polymerase. Amplification conditions were as follows: 30 cycles of 98°C for 10 s, 58°C for 30 s and 72°C for 1 min. The PCR products were purified using MultiScreen PCRμ96 plates (Millipore), according to the manufacturer’s instructions. The size of the PCR products was assessed by electrophoresis. The agarose gels were stained with ethidium bromide and visualized under UV light.

Selection of restriction enzymes and PCR-RFLP analysis

Based on the DNA sequence information obtained in this study, we surveyed the restriction sites of the SBE locus extensively using Geneious Pro 7.1.5 (Biomatters Ltd.). The restriction enzymes with digestion sites that were conserved within a species and variable among other species in a given sequence were selected. The intron 11 of the SBE gene was used for the identification of A. cruentus. A fragment from the SBE gene of 278 bp (position 3,536 to 3,240) containing an MseI restriction site was amplified by PCR using the primers crsbe-F: 5’-AGCGAATTGCGACGAATTATGTTA CAT-3’ and crsbe-R: 5’-TTCCTTTTCCACCGAACATCAA TGCAT-3’. PCR conditions were as follows: 30 cycles of 98°C for 10 s, 55°C for 30 s and 72°C for 30 s. PCR products were digested with the MseI (RspRSII) restriction enzymes (Takara) in a total volume of 20 μl at 60°C for 1 h based on the manufacturer’s instructions, with some modifications. The digested fragments were separated in 2% agarose gels by electrophoresis in TBE buffer for approximately 45 min and visualized by staining with ethidium bromide.

Sequence analyses

The DNA sequences of the amplified products were determined in both directions using the BigDye Terminator Cycle Sequencing Kit (version 3.1, Applied Biosystems) on an ABI 3130xl Genetic Analyzer (Applied Biosystems). The sequencing primer (3.2 pmol) and dye terminator ready-reaction sequencing premix (8 μl) were added to each template. Following a denaturation step at 96°C for 2 min, the dye terminator reaction was performed for 25 cycles of 96°C for 15 s, 50°C for 1 s and 60°C for 4 min. A multiple sequence alignment and analyses of the deduced amino acid and nucleotide sequences were performed using ClustalW 2.1 as a module of Geneious Pro 7.0.5 (Biomatters). Polymorphic site candidates were identified using CodonCode Aligner 4.2.5 (CodonCode Co., Dedham, MA, USA).

Results and Discussion

Previous analyses of the genetic relationships in the genus Amaranthus have used several techniques, including the chromosome number and hybrid fertility (Gupta and Gudu 1991, Pal and Khoshoo 1974), isozymes (Chan and Sun 1997, Hauptli and Jain 1984), random amplified polymorphic DNAs (Chan and Sun 1997, Das 2012, Transue et al. 1994), restriction-site variation of chloroplasts and nuclear DNAs (Lanoue et al. 1996), DNA fingerprints (Sun et al. 1999), amplified fragment-length polymorphisms and inter-sequence simple repeats (Xu and Sun 2001), micromorphology (Costea et al. 2006), microsatellite markers (Mallory et al. 2008) and protein markers (Džunková et al. 2011). However, most of these studies focused on genetic diversity and/or evolutionary relationships among the cultivated species and their wild ancestors. We therefore wanted to provide a rapid molecular technique to distinguish among the cultivated grain species that are typically widely used around the world. Recently, molecular techniques, based on PCR-RFLP marker analysis, were developed for the identification of two cultivated species, A. caudatus and A. hypochondriacus (Park and Nishikawa 2012b). This was the first study using molecular techniques to identify species among the cultivated grain amaranths within the genus Amaranthus. The use of molecular techniques for species identification is very uncommon for this crop and a rapid molecular technique to identify the A. cruentus species is required.

In this study, we first developed a PCR-RFLP marker, which was able to identify the A. cruentus species, by comparing SBE locus sequences among the grain amaranth
Table 1. Summary of sampled the cultivated grain amaranth accessions and their polymorphism in species-specific sites from the SBE locus

| Species                  | No.   | Accession no. | Origin          | T-C polymorphism in intron 11 of the SBE locus |
|--------------------------|-------|---------------|-----------------|-----------------------------------------------|
| **A. cruentus**          | cr1   | Ames 22000    | Guatemala       | T                                             |
|                          | cr2   | Ames 22004    | Guatemala       | T                                             |
|                          | cr3   | Ames 5676     | Guatemala       | T                                             |
|                          | cr4   | PI 511715     | Guatemala       | T                                             |
|                          | cr5   | PI 511718     | Guatemala       | T                                             |
|                          | cr6   | Ames 5165     | United States   | T                                             |
|                          | cr7   | Ames 5318     | United States   | T                                             |
|                          | cr8   | Ames 5677     | United States   | T                                             |
|                          | cr9   | Ames 5480     | Mexico          | T                                             |
|                          | cr10  | Ames 15189    | Mexico          | T                                             |
|                          | cr11  | PI 451710     | Mexico          | T                                             |
|                          | cr12  | PI 490662     | Mexico          | T                                             |
|                          | cr13  | PI 517226     | Mexico          | T                                             |
|                          | cr14  | PI 576481     | Mexico          | T                                             |
|                          | cr15  | PI 604558     | Mexico          | T                                             |
|                          | cr16  | PI 511713     | Peru            | T                                             |
|                          | cr17  | Ames 1977     | India           | T                                             |
|                          | cr18  | Ames 2037     | India           | T                                             |
|                          | cr19  | PI 566897     | India           | T                                             |
|                          | cr20  | PI 576448     | Nigeria         | T                                             |
|                          | cr21  | PI 536996     | Ghana           | T                                             |
|                          | cr22  | Ames 1968     | Zaire           | T                                             |
|                          | cr23  | Ames 5369     | Zaire           | T                                             |
|                          | cr24  | PI 494774     | Zambia          | T                                             |
| **A. caudatus**          | ca1   | Ames 15176    | Argentina       | C                                             |
|                          | ca2   | Ames 15177    | Argentina       | C                                             |
|                          | ca3   | Ames 15179    | Argentina       | C                                             |
|                          | ca4   | PI 481607     | Bhutan          | C                                             |
|                          | ca5   | PI 490604     | Bolivia         | C                                             |
|                          | ca6   | PI 490607     | Bolivia         | C                                             |
|                          | ca7   | PI 568139     | Bolivia         | C                                             |
|                          | ca8   | PI 568153     | Bolivia         | C                                             |
|                          | ca9   | PI 166107     | India           | C                                             |
|                          | ca10  | PI 175039     | India           | C                                             |
|                          | ca11  | Ames 10176    | Pakistan        | C                                             |
|                          | ca12  | PI 490614     | Peru            | C                                             |
|                          | ca13  | PI 490621     | Peru            | C                                             |
|                          | ca14  | PI 490626     | Peru            | C                                             |
|                          | ca15  | PI 568139     | Peru            | C                                             |
|                          | ca16  | PI 490639     | Peru            | C                                             |
|                          | ca17  | PI 516853     | Peru            | C                                             |
|                          | ca18  | PI 516863     | Peru            | C                                             |
|                          | ca19  | PI 516893     | Peru            | C                                             |
|                          | ca20  | PI 511705     | Peru            | C                                             |
|                          | ca21  | PI 511705     | Peru            | C                                             |
|                          | ca22  | IB 85-3291*   | Nepal           | C                                             |
| **A. hypochondriacus**   | hy1   | Ames 5436     | Mexico          | C                                             |
|                          | hy2   | Ames 5467     | Mexico          | C                                             |
|                          | hy5   | Ames 5132     | Mexico          | C                                             |
|                          | hy8   | PI 477917     | Mexico          | C                                             |
|                          | hy9   | PI 560476     | Mexico          | C                                             |
|                          | hy10  | PI 490755     | Mexico          | C                                             |
|                          | hy11  | PI 564560     | Mexico          | C                                             |
|                          | hy12  | PI 560794     | Mexico          | C                                             |
|                          | hy13  | Ames 5158     | Puerto Rico     | C                                             |
|                          | hy14  | Ames 5689     | Brazil          | C                                             |
|                          | hy15  | Ames 5355     | Chile           | C                                             |
|                          | hy16  | Ames 21766    | China           | C                                             |
|                          | hy17  | PI 542595     | China           | C                                             |
|                          | hy18  | PI 590891     | China           | C                                             |
|                          | hy19  | PI 337611     | Uganda          | C                                             |
|                          | hy20  | Ames 1972     | Nigeria         | C                                             |
|                          | hy21  | Ames 1975     | Nigeria         | C                                             |
|                          | hy22  | PI 558499     | United States   | C                                             |
|                          | hy23  | PI 274229     | India           | C                                             |
|                          | hy24  | 85-10-10-3-15*| India           | C                                             |
|                          | hy25  | Almora*       | India           | C                                             |
|                          | hy26  | 85-10-27-3-5* | India           | C                                             |
|                          | hy27  | AC#00406*     | Sri Lanka       | C                                             |
|                          | hy28  | PI 540446     | Pakistan        | C                                             |
|                          | hy29  | Ames 5609     | Afghanistan     | C                                             |
|                          | hy30  | BU 95007*     | Bhutan          | C                                             |
|                          | hy31  | Ames 5660     | Zambia          | C                                             |
|                          | hy32  | TMN-638*      | Nepal           | C                                             |
|                          | hy33  | TMN-647*      | Nepal           | C                                             |
|                          | hy34  | SU87-871478*  | Nepal           | C                                             |

* The collection in Shinshu University, Japan.
PCR-RFLP marker for the identification of *A. cruentus* species. We determined the partial *SBE* genomic sequence in 72 accessions of the cultivated grain amaranths by direct sequence analysis. The alignments of the 72 *SBE* sequences produced a matrix of 7,453 bp. Comparisons of the aligned *SBE* sequences revealed several substitutions and insertions/deletions. On the basis of DNA sequence data, the digestion patterns were predicted for various restriction enzymes using Geneious Pro 7.0.5 software. Finally, the *Mse*I enzyme was selected to achieve the best species-specific pattern for identification of *A. cruentus*. The sequence data for the *SBE* locus in all *A. cruentus* accessions contained 5′-T/T-3′ in intron 11, while the other two species, *A. caudatus* and *A. hypochondriacus* contained 5′-T/C-3′ in intron 11 (Fig. 1). This result indicated that the *SBE* gene is highly conserved and, consequently, a good molecular marker for diagnostic studies. Thus, the comparative analysis of *SBE* sequences from 72 amaranth accessions provided the basis for the design of diagnostic primers having the potential for the species-specific identification of *A. cruentus* by the PCR-RFLP method. In this study, we designated this one-base substitution as the “T-C polymorphism” (Table 1).

### Table 2. Primer sequences and annealing temperatures used for amplification of fragments from *SBE* locus

| Fragment | Primer pairs | Forward and reverse PCR primer sequences (5′→3′) | Amplified region | Expected length | Annealing temperature |
|----------|--------------|-------------------------------------------------|-----------------|----------------|----------------------|
| 1        | SBEg-F3/SBEg-R3 | F: TGCAGCACCCTATGCAAGATATGCACTGGAGGAAACAGGCTTAT CAGGCTTATGCAAGATATGCACTGGAGGAAACAGGCTTAT | partial exon 5–partial exon 6 | 853 | 58°C |
| 2        | SBEg-F4/SBEg-R4 | F: ATGGGGCTAGCCTTAGATGCAAGATATGCACTGGAGGAAACAGGCTTAT CAGGCTTATGCAAGATATGCACTGGAGGAAACAGGCTTAT | partial exon 6–partial exon 7 | 924 | 58°C |
| 3        | SBEg-F5/SBEg-R5 | F: AGTGGAGGGAATTCTGCTTCAATACTGGAGGAAACAGGCTTAT CAGGCTTATGCAAGATATGCACTGGAGGAAACAGGCTTAT | partial exon 7–partial exon 8 | 954 | 58°C |
| 4        | SBEg-F6/SBEg-R6 | F: AGGCTACTTAAACTTATGCAAGATATGCACTGGAGGAAACAGGCTTAT CAGGCTTATGCAAGATATGCACTGGAGGAAACAGGCTTAT | partial exon 8–partial exon 9 | 1,134 | 58°C |
| 5        | SBEg-F7/SBEg-R2 | F: ATGGGAATCTTCTTCTGTGCAAGATATGCACTGGAGGAAACAGGCTTAT CAGGCTTATGCAAGATATGCACTGGAGGAAACAGGCTTAT | partial exon 9–partial exon 10 | 1,124 | 58°C |
| 6        | SBEg-F8/SBEg-R8 | F: ATGGGAATCTTCTTCTGTGCAAGATATGCACTGGAGGAAACAGGCTTAT CAGGCTTATGCAAGATATGCACTGGAGGAAACAGGCTTAT | partial exon 10–partial exon 11 | 936 | 58°C |
| 7        | SBEg-F9a/SBEg-R3 | F: TGCAGCATTGGTTCTCCTGCAATGGGGAAACAGGCTTAT CAGGCTTATGCAAGATATGCACTGGAGGAAACAGGCTTAT | partial exon 12–exon 14 | 1,181 | 58°C |

Fig. 1. Partial sequence alignment of the *SBE* locus from *A. cruentus*, *A. caudatus* and *A. hypochondriacus*. Solid black box shows the species-specific restriction cleavage site for the enzyme *Mse*I. Major SNP, T-C polymorphism is underlined. Shaded area is partial exon 12.

Next, we examined the genetic variation in intron 11 of the *SBE* locus from 72 accessions using the PCR-RFLP method (Fig. 2). The primer set crsbe-F/crsbe-R successfully amplified a control region using DNA extracts from all samples. This PCR product, located from 3,240-bp (intron 11) to 3,536-bp (exon 12), was approximately 278 bp (Fig. 2a). After restriction enzyme digestion, the results indicated that PCR-RFLP was a suitable tool for identifying *A. cruentus* accessions. As shown in Fig. 2b, digestion of the control region in *A. cruentus* by *Mse*I produced two fragments, 174 bp and 104 bp, whereas *A. caudatus* and *A. hypochondriacus* produced the original PCR fragment of approximately 278 bp. This result indicated that the fragment of *A. cruentus* species contained an *Mse*I site, while the fragments of the other two amaranths had no *Mse*I sites. Thus, our results clearly showed that this PCR-RFLP method was highly reliable for identifying *A. cruentus* from among the cultivated grain amaranths. Finally, the PCR-RFLP method developed here will save a significant amount of time and reagents when identifying the *A. cruentus* species within the cultivated grain amaranths.
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Fig. 2. PCR-RFLP method to identify *A. cruentus*. a. A single 278-bp fragment was amplified from three cultivated grain species of *Amaranthus* using primers specific for the SBE locus (see Materials and Methods for details). Markers represent a 100-bp DNA ladder. b. Schematic and result of PCR-RFLP for identifying *A. cruentus* using intron 11 of the SBE locus. Restriction profiles of PCR amplification of intron 11 of SBE followed by digestion with *Mse* I. Restriction enzyme cleavage site is shown in bold, and one-base substitution, T-C polymorphism is underlined. Markers represent a 100-bp DNA ladder.