

# Development of multiplex PCR for species-specific identification of the *Poaceae* family based on chloroplast gene, *rpoC2*

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**Abstract** In this study, we report the development of a multiplex polymerase chain reaction (PCR) method using species-specific primers for the simultaneous detection of *Poaceae* members, including adlay, barley, maize, rice, and wheat, based on sequence polymorphism in DNA-directed RNA polymerase beta-prime chain genes (*rpoC2*). Species-specific primer pairs were constructed with a common forward primer and reverse primers differing by sequence polymorphisms and gene location. Each primer pair was designed to PCR-amplify products of five chloroplast genes of 443, 346, 278, 221, and 96 bp for rice, barley, adlay, wheat, and maize, respectively. Multiplex PCR with a series of template DNA concentrations (0.01–10 ng/μL) was used to optimize amplification of fragments from pooled *Poaceae*. In addition, species-specific primers were used to detect components of seven commercial flour-mixed products. This combination of the sensitivity of multiplex PCR with the specificity of primers designed to detect unique species has broad applications in the processed food industry.

**Keywords** Commercial flour-mixed product · cpDNA · Multiplex PCR · *Poaceae* family · Species-specific primer

## Introduction

Food consumerism now emphasizes convenience for diverse lifestyles. Many commercial health foods have been developed using mixed grain powders of various cereal crops. *Sunsik* is a cereal food product derived from a ready-to-eat powder that consists of roasted grains, nuts, and dried vegetables (Koh et al. 2014), and can be consumed without cooking. High-quality *Sunsik* is required in various compositions for different niche markets (e.g., baby food), but possible contaminants threaten its marketability.

Commercial fraud is presently a great concern to regulatory authorities and consumers, especially the mislabeling or incorrect labeling of allergenic constituents such as wheat gluten. Therefore, analytical techniques have been developed for the analysis of food contents. Such highly specific and sensitive techniques based on polymerase chain reaction (PCR) have proven useful for identifying origins of foods. These PCR-based methods are rapid, low cost (Mafra et al. 2008), and have been applied successfully to species identification of legumes (Weder 2002), cereals (Terzi et al. 2005), nuts (Poms et al. 2004), olive oil (Breton et al. 2004; Pasqualone et al. 2004), and grape (Faria et al. 2000; Rodriguez-Plaza et al. 2006). In addition, many studies have focused on methods to detect genetically modified organisms in soybean- and maize-derived food products.

Multiplex PCR can simultaneously amplify two or more targets in the same reaction, and is reliable, flexible, and economic. Multiplex PCR has been applied successfully for species identification in foods of various origins, such as meat (Matsunaga et al. 1999; Rodriguez et al. 2001; Lopez-Andreo et al. 2006), fish (Trotta et al. 2005), and seafood (Trotta et al. 2005; Santaclara et al. 2006) products. In addition, many studies have reported the use of

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multiplex PCR as a rapid and convenient screening assay for the detection of GM crops, such as soybean, maize, and canola (James et al. 2003; Germini et al. 2004; Forte et al. 2005). Even though it has many advantages, multiplex PCR also has many challenges, such as self-inhibition among primer sets, low amplification efficiency, and lack of efficiency in different templates for broad application (Xu et al. 2012).

Chloroplasts are organelles found only in plant and algal cells and play an important role in photosynthesis. Most chloroplast genomes (cpDNA) form a single ring from 120 to 170 kb (Clegg et al. 1994; Shaw et al. 2007). The haploid cpDNA has a simple and stable genetic structure and shows little to no recombination. Chloroplast genes such as *atpF-H*, *marK*, *psbK-1*, *rbcL*, *rpoB*, *rpoC1*, and *trnH-psbA* have been used to develop molecular markers to distinguish among plant species (Kress et al. 2005; Newmaster et al. 2006; Chase et al. 2007; Seberg and Petersen 2009; Hollingsworth et al. 2011). Hwaang et al. (2015) reported that the *rpoC2* gene used for the development of a chloroplast marker for detecting rice in food ingredients. This study suggested that the *rpoC2* gene was an effective chloroplast marker because of high sequence variation and low genetic variation in plant species.

In this study, we report a multiplex PCR method to simultaneously detect five members of *Poaceae*, i.e., adlay, barley, maize, rice, and wheat. Specific primers for each species were developed based on sequence polymorphisms in chloroplast *rpoC2*, and multiplex PCR was applied to detect components of commercial flour-mixed products such as *Sunsik*.

## Materials and methods

### Sequence analysis of cpDNA and primer design

The complete chloroplast genome sequences of five grass species, including adlay (*Coix lacryma-jobi*), maize (*Zea mays*), rice (*Oryza sativa* subsp. *japonica*), barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*) were downloaded from CpBase database (<http://chloroplast.ocean.washington.edu/>). The sequences were aligned using ClustalW2 (<ftp://ebi.ac.uk/pub/software/clustalw2/>). *rpoC2* sequences were aligned using ClustalW2, and VectorNTI (Invitrogen™, Life Technologies, USA) was used to design specific primer pairs from *rpoC2*, which is shown to have high sequence polymorphism among the five plant species. Each reverse primer was designed to amplify a region of cpDNA with sequence polymorphism specific to one of the five plant species and a common forward primer was designed to target a conserved sequence area determining size differences in the PCR amplicons.

### Sample preparation

Seeds of adlay, barley, maize, rice, and wheat were provided by the RDA-Genebank (Rural Development Administration, Korea). Commercial flour-mixed products (*Sunsik*) were purchased at local stores (Table 1).

### Genomic DNA extraction

Plants were grown in a growth chamber at 25 °C for 2 weeks with a photoperiod 16/8 h (light/dark) and 70 %

**Table 1** Food material of seven commercial flour-mixed products used in the study

Food makers	Product name	Material indication	
		Main component (%)	Additional ingredients
A	Roasted and ground adlay grains	Adlay (100)	
B	Powder made of mixed grains	Barley (37.2), Rice (20)	Maize, wheat, soybean, azuki, adlay, sesame, sorghum, glutinous millet
C	Meal of the day	Adlay (30), Barley (20), Rice (20)	Soybean, maize, sesame, licorice
D	Powder made of mixed grains	Barley (14.2), Rice (14.1)	Soybean, maize, wheat, peanut, sunflower seed, almond, sesame
E	Powder of germinated three cereals	Rice (30)	Soybean, barley, peanut, maize, chestnut, adlay, sorghum, millet, sesame, kelp, spinach, green tea, sea mustard, pumpkin, broccoli, chlorella, walnut, perilla, ginkgo nut, safflower seed, jujube, carrot, angelica utilis, kale, chinese yam, balloon flower, onion, apple, cabbage, mugwort, anchovy, shiitake mushroom, pine needle, potato, sweet potato, mulberry leaf
F	Powder made of mixed germinated brown rice	Barley (30), Rice (N/A)	Soybean, maize, sorghum, glutinous millet, adlay, millet, sesame
G	Powder made of mixed grains	Rice (55.6), Barley (29), Quinoa (5)	Maize, soybean, adlay, sesame, millet

humidity. Total genomic DNA (gDNA) was extracted from mature leaves and flour-mixed products with an i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology, Korea) according to the manufacturer's protocol. Quantity and quality of extracted DNA were measured with a Qubit<sup>®</sup> 2.0 Fluorometer (Invitrogen<sup>™</sup>, Life Technologies) using the Qubit<sup>®</sup> dsDNA BR Assay Kit (Invitrogen<sup>™</sup>, Life Technologies) according to the manufacturer's protocol. For total DNA extraction, we prepared 200 mg of each commercial flour-mixed products. The total DNA concentration of commercial flour-mixed products was 140–10 ng/μL, and it was diluted to 10 ng/μL for multiplex PCR.

### PCR conditions

PCR analyses were carried out on a C1000 Thermal Cycler (BIO-RAD<sup>™</sup>, USA). The efficiency of primer pairs amplifying the target sequences was tested by simplex PCR using the corresponding target gDNA. For testing primer efficiency in multiplex PCR, each primer pair was added into five different gDNA concentrations (e.g., 10, 1, 0.1, 0.01, and 0.001 ng). All simplex and multiplex PCR assays were performed in a final volume of 25 μL with the following concentrations of components: 10 ng template DNA, 10 × buffer, 25 mM MgCl<sub>2</sub>, 2.5 mM dNTPs, and 0.5 U/μL *Taq* DNA polymerase (Takara Bio Company, Japan). The amplification conditions were as follows: an initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 47 °C for 30 s, primer extension at 72 °C for 30 s, and final extension at 72 °C for 7 min. Simplex and multiplex PCRs had identical amplification conditions.

### Analysis of PCR products

PCR products were analyzed by agarose gel electrophoresis. The gel was prepared with 1.5 % Seakem LE agarose (Lonza, USA) for simplex and multiplex PCR and visualized using a gel doc imaging system (BIO-RAD<sup>™</sup>).

## Results and discussion

### Species-specific primer design

To analyze species-specific sequences in members of *Poaceae*, chloroplast genome sequences of adlay, barley, maize, wheat, and rice were downloaded from the CpBase database. The sizes of *rpoC2* in these five grass species were 4563, 4434, 4584, 4440, and 4542 bp, respectively. This gene showed high sequence polymorphism across the five plant species. The coding region of *rpoC2* possesses extra coding

sequence specific to the *Poaceae* family, which is composed of tandem repeats in a complex arrangement with a large number of insertion/deletion (indel) events (Cummings et al. 1994). Thus, this gene is an excellent candidate for distinguishing specifically among members of *Poaceae*.

Design of species-specific primers was based on an *rpoC2* multispecies sequence alignment of the five species (Fig. 1). We first designed one common forward primer (5'-CGCGATTTTCCTAAGAATT-3') to be shared among five species. Second, five species-specific primers were designed to target sequence polymorphisms and differences in amplicon sizes (Table 2; Fig. 1). Each primer had an optimal length of 18 nucleotides.

### Multiplex PCR using plant genomic DNA

To verify primer specificity, simplex PCRs were carried out on each template gDNA extracted from *Poaceae* family members including adlay, barley, maize, rice, and wheat. Each primer pair generated specific fragments of 443 bp for rice, 346 bp for barley, 278 bp for adlay, 221 bp for wheat, and 96 bp for maize (Fig. 2, Line A–E). When multiplex PCR was carried out on pooled DNA of five species, the primer sets retained identical specificity (Fig. 2, Line F), indicating that the primer sets are sensitive and highly reproducible. Each primer concentration was serially diluted (5–30 pmol) to determine the absolute sensitivity of the multiplex PCR and suitable concentrations are shown in Table 2. In addition, each of the PCR products were sequenced to confirm amplicon identity with the reference sequence using the ClustalW2 program. Figure 3 shows a perfect match between the sequenced PCR products and the reference sequences.

In a previous study (Ronning et al. 2005), differentiation among *Poaceae*, including rye, barley, oat, rice, and maize was obtained by amplification of the chloroplast *trnL* intron sequence with universal primers, followed by a cyclic labeling of oligonucleotide probes and subsequent hybridization to an oligonucleotide microarray. While this method may offer high sensitivity applicable in the routine diagnosis of food samples (Ronning et al. 2005), a limitation of this protocol is the expensive equipment and costs associated with its application. Our results indicate that the species-specific primers designed to target sequence polymorphisms in a multispecies sequence alignment can be combined in multiplex PCR to amplify five cereal genes simultaneously. Major advantages of this multiplex PCR assay are that it can serve as a rapid and cost-effective diagnostic.

To investigate the sensitivity of multiplex PCR, PCR was performed on mixed template DNA samples that were serially diluted (10<sup>-3</sup> to 10 ng/μL) and pooled (Fig. 4). The



**Fig. 1** A *rpoC2* gene sequence alignment of five *Poaceae* family species, including adlay, barley, wheat, rice, and maize. The box indicates a common forward primer sequence. Species-specific primer sites of five species are marked with each under dot line

**Table 2** Summary of five species-specific primer for species distinction in *Poaceae* family

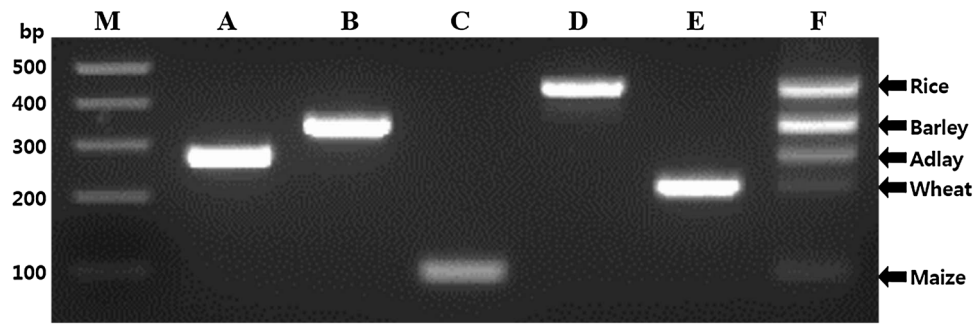
Cereal	Primer sequence (5'-3')	Orientation	Amplicon size (bp)	Product positions		Concentration (pmol)	G+C (%)	Tm (°C)
				Start	End			
Adlay	ACCGCATTGGGATATTC	Reverse	278	3013	3290	20 <sup>a</sup> 20 <sup>b</sup>	44	46
Barley	TACTAGCTACAATAGCCC	Reverse	346	2971	3316	5 <sup>a</sup> 10 <sup>b</sup>	44	46
Maize	CCTATTATTGGGAATCAA	Reverse	96	3034	3129	30 <sup>a</sup> 20 <sup>b</sup>	33	41
Rice	TTGTCAAGGAGCAAATGC	Reverse	443	2977	3419	10 <sup>a</sup> 10 <sup>b</sup>	44	46
Wheat	GACAAAATCATCAAAGAG	Reverse	221	2923	3143	20 <sup>a</sup> 10 <sup>b</sup>	33	41
	CGCGATTTTCCTAAGAATT	Forward <sup>c</sup>				10 <sup>a</sup> 20 <sup>b</sup>	39	43

<sup>a</sup> Used to simplex PCR

<sup>b</sup> Used to multiplex PCR

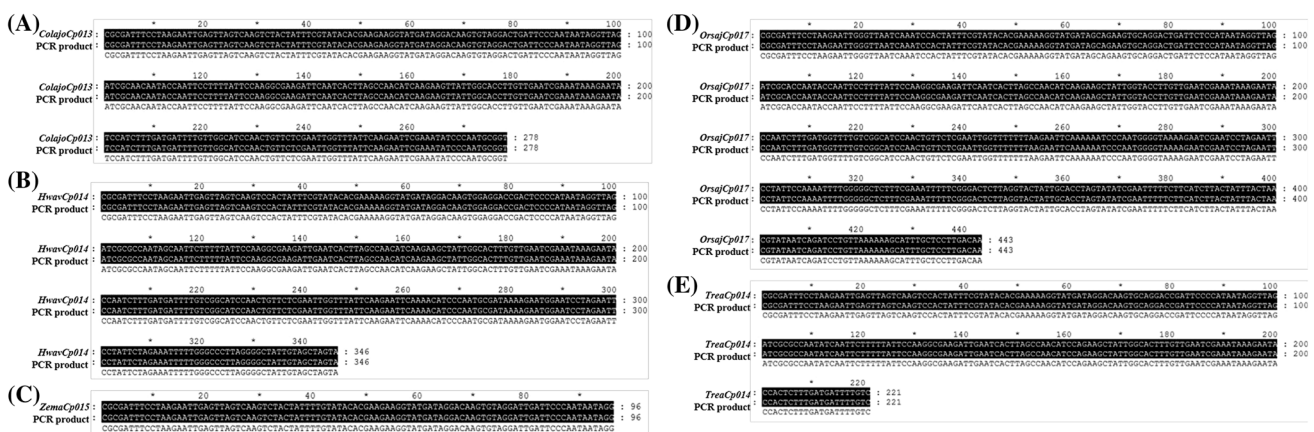
<sup>c</sup> Shared all PCR reactions





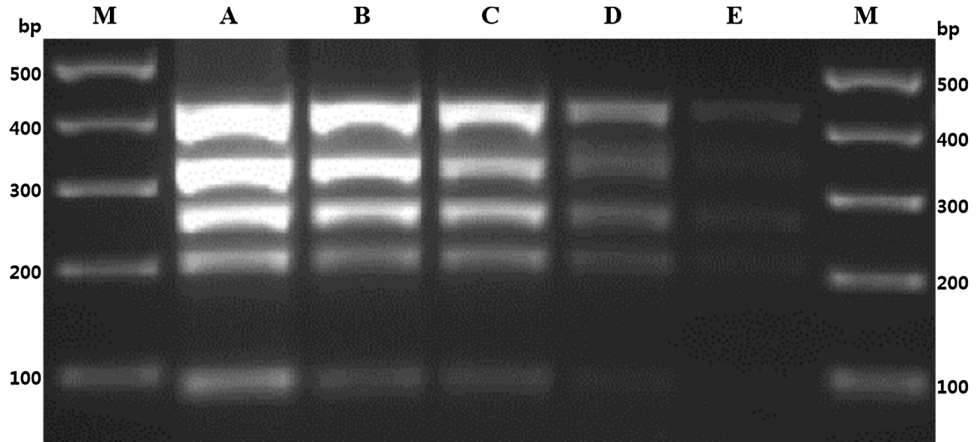
**Fig. 2** Specificity of species-specific primer available for identification of *Poaceae* family. Amplified PCR products of 443, 346, 278, 221, and 96 bp correspond to the rice, barley, adlay, wheat, and maize, respectively. Line M, 1 kb DNA ladder marker; *Line A* simplex PCR of Adlay specific primer; *Line B* simplex PCR of Barley specific

primer; *Line C* simplex PCR of Maize specific primer; *Line D* simplex PCR of Rice specific primer; *Line E* simplex PCR of Wheat specific primer; *Line F* multiplex PCR of combination of species-specific primer



**Fig. 3** The sequence analysis of PCR products with a reference sequence via the ClustalW2. (A) Adlay; (B) Barley; (C) Maize; (D) Rice; (E) Wheat

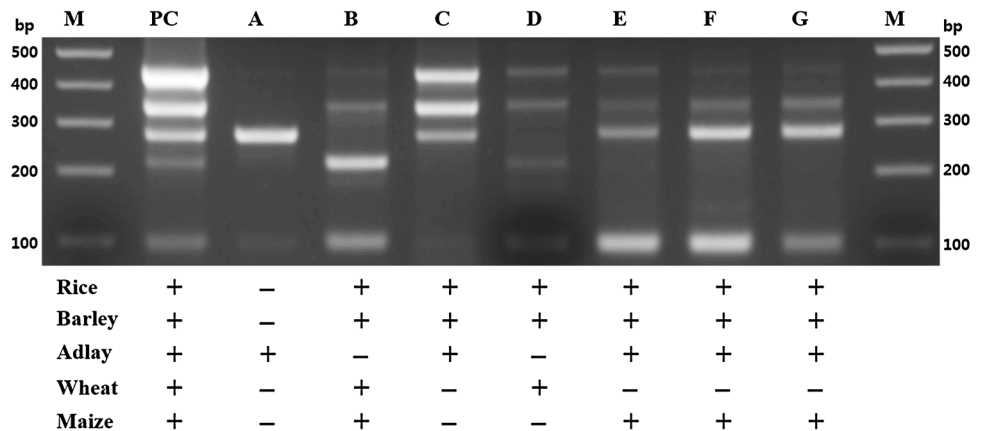
**Fig. 4** Sensitivity of species-specific primer available for identification of the *Poaceae* family with difference concentrations of template genomic DNAs. *Line M* 1 kb DNA ladder marker; *Line A* 10 ng; *Line B* 1 ng; *Line C* 0.1 ng; *Line D* 0.01 ng; *Line E* 0.001 ng



sensitivity of the species-specific primers was demonstrated by the detection of various concentrations of template DNA. Products amplified with the  $10^{-3}$  ng DNA concentration

were present but weak, whereas products with template concentrations of  $10^{-2}$  to 10 ng were clearly visible. For reproducible results, all tests were performed in triplicate.

**Fig. 5** Multiplex PCR identification of seven commercial flour-mixed products (*Sunsik*) using species-specific primer pairs. *Line M* 1 kb DNA ladder marker; *Line PC* multiplex PCR of combination of species-specific primer with template DNA of five species; *Line A–G* purchased commercial flour-mixed products (see as Table 1). “+” indicates detection of each cereal by multiplex PCR with species-specific primer



### Multiplex PCR of commercial flour-mixed products (*Sunsik*)

*Sunsik*, a powdered mixture of roasted grains and other food materials, is widely consumed as a meal and energy substitute in Korea. To validate the feasibility of multiplex PCR in commercial flour-mixed products (*Sunsik*), extracted DNA from seven *Sunsik* products purchased from local stores was subjected to multiplex PCR with the species-specific primers. We found that all seven samples amplified clear PCR products consistent with the materials comprising these commercial flour-mixed products (Fig. 5; Table 1). For example, “powder made of mixed grains” of food maker B includes barley and rice (main component), maize, wheat, adlay, and additional ingredients. Our species-specific primers detected all grass species (including rice, barley, wheat, and maize) except adlay, which may indeed be a commonly mislabeled species. The results of this assay demonstrate the specificity and sensitivity of species-specific primers to identify commercial flour-mixed products. Adulteration of food products with contaminant species is a significant problem for governmental regulatory affairs, the food industry, and consumers themselves. Commercial flour-mixed products (e.g., *Sunsik*) are produced in many forms, such as roasted cereal and as a vegetable. Therefore, a rapid and high-throughput method for species-specific identification of pure *Sunsik* is desirable to avoid improper labeling. In this study, we report an efficient multiplex PCR method to simultaneously detect five species of cereal at varying concentrations. Each primer showed no cross-reactivity within five species and amplified template DNA from 10 to 0.001 ng/μL.

In conclusion, the plant chloroplast genome, present in multiple copies within a cell, is often used to discriminate among plant species because of its intraspecies consensus and interspecies variability (Li et al. 2012). To test whether a PCR method based on a chloroplast gene would be more

sensitive for identification of species, we designed a pair of species-specific primers targeting *rpoC2* to (1) simultaneously amplify in five plant species and to (2) discriminate plant species in *Sunsik*. Our species-specific primer combinations for multiplex PCR are able to identify and discriminate five cereals including commercial flour-mixed products. Although five primer pairs were included in the experiment, more species-specific primers can be developed to include a wider range of cereals in the future. More specific primers have the potential to detect more plant species that constitute commercial flour-mixed products. Therefore, combining multiplex PCR with species-specific primers should prove to be a powerful tool for food product authentication.

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