Specific Detection of Potentially Allergenic Peach and Apple in Foods Using Polymerase Chain Reaction

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ABSTRACT: Two PCR methods were developed for specific detection of the trnS-trnG intergenic spacer region of *Prunus persica* (peach) and the internal transcribed spacer region of *Malus domestica* (apple). The peach PCR amplified a target-size product from the DNA of 6 *P. persica* cultivars including 2 nectarine and 1 flat peach cultivar, but not from those of 36 nontarget species including 6 *Prunus* and 5 other Rosaceae species. The apple PCR amplified a target-size product from the DNA of 5 *M. domestica* cultivars, but not from those of 41 nontarget species including 7 Maloideae and 9 other Rosaceae species. Both methods detected the target DNA from strawberry jam and cookies spiked with peach and apple at a level equivalent to about 10 μg of total soluble proteins of peach or apple per gram of incurred food. The specificity and sensitivity were considered to be sufficient for the detection of trace amounts of peach or apple contamination in processed foods.

KEYWORDS: *food allergy, peach, apple, trnS-trnG intergenic spacer, internal transcribed spacer (ITS), polymerase chain reaction*

■ INTRODUCTION

Peach and apple are known to cause allergic reactions in certain populations.[1,2] The presence of undeclared apple or peach poses a risk to consumers allergic to these fruits. Thus, the methods for detecting trace amounts of peach or apple in food products should be of value to the food industries and regulatory agencies for reducing chances of unexpected exposure of the allergic consumers to the offending foods.

Peach and apple allergens belong to mainly four protein families: pathogenesis related (PR) 10 proteins, thaumatin-like proteins, lipid transfer proteins (LTP), and profilins.[3–5] Because of the occurrence of homologous proteins, wide cross-reactivity among fruits, especially those of the family Rosaceae, has been documented.[6–8] However, a patient who has developed an allergy to a particular fruit does not necessarily become clinically allergic to all other fruits having homologous and immunologically cross-reactive proteins. For example, Rodriguez et al. reported that 10 in 22 peach-allergic patients did not show clinical responses to any of the Rosaceae fruits tested, including apple, apricot, almond, plum, strawberry, and pear.[7] Therefore, a peach detection method that can differentiate peach from other Rosaceae fruits should benefit peach-allergic patients by reducing unnecessary avoidance of foods containing Rosaceae fruits other than peach.

Cultivars of peach belong to the species *Prunus persica*, which includes nectarine (*P. persica* var. *nucipersica*) and flat peach (*P. persica* var. *platycarpa*). Because accumulation of the major peach allergens, especially Pru p 3, has been confirmed not only in peach but also in nectarine and flat peach,[9–12] we chose *P. persica* including nectarine and flat peach as the target for our peach detection method.

For our apple detection method, we chose *Malus domestica* as the target, because it is the species of domesticated apple cultivated widely on all continents except Antarctica,[13,14] and its cultivars have been confirmed to possess allergenicity, although of various degrees.[15] Because no reports on their allergenicity could be found, and the chances that they would find their way into food products were thought to be slim, most of the "wild apples" or "crab apples" belonging to *Malus* spp. other than *M. domestica* and a few of its very close wild relatives were excluded from the target.

Among the currently available allergen detection methods, polymerase chain reaction (PCR)-based methods are highly specific and sensitive and are suitably used as confirmatory methods for positive ELISA tests to exclude false positives,[16,17] although they do not detect allergenic proteins per se. ELISA methods are sensitive, quantitative, and easy to perform and are especially suited as screening tests, but their possible cross-reactivity with homologous nontarget proteins may lead to false-positive results. Although ELISA methods for the quantitation of peach LTP (Pru p 3) and apple LTP (Mal d 3) have already been reported,[18,19] PCR-based methods should still be of value for confirmation of the positive results.

Under the Japanese labeling system for foods containing allergens, declarations of 7 food items are mandatory and those of 18 food items including peach and apple are recommended. The threshold for monitoring the mandatory labeling by ELISA is established at 10 μg protein/g food.[20] An ELISA result indicating the presence of total soluble proteins of an allergenic ingredient in the test sample at or >10 ppm (μg/g or μg/mL) is deemed positive, and, unless the presence of such allergenic

ingredient is evident from the production records, a confirmation test is performed. Therefore, the PCR-based methods are expected to have a sensitivity equivalent to a contamination level of at least 10 ppm total soluble protein of the target allergenic ingredient.

For the PCR-based methods, target DNA sequences may be conveniently selected from the regions commonly employed in the molecular phylogeny studies (barcode gene regions), such as the trnS-trnG intergenic spacer, internal transcribed spacer (ITS), rbcL, trnL intron, matK, and psbA, to name a few. The abundance of interspecific sequence variations in these regions and the wealth of readily accessible sequence data for a wide variety of species make the target specific primer design easy to accomplish. Moreover, the multicopy nature of the sequences in these regions is helpful in making the detection methods achieve the required sensitivity.[21−24] Here, we report two PCR-based methods for the specific detection of peach (*P. persica*) and apple (*M. domestica*) using primer pairs designed on the trnS-trnG intergenic spacer (trnS-trnG IGS) region and the internal transcribed spacer-1 (ITS-1) region, respectively.

■ MATERIALS AND METHODS

Plant Materials. The fruits of three peach cultivars (*P. persica* cv. Hakuho, cv. Kawanakajima hakuto, and cv. Golden peach), two nectarine cultivars (*P. persica* var. *nucipersica* cv. Flavortop and cv. Shuho), one flat peach cultivar (*P. persica* var. *platycarpa* cv. Da Hong Pan Tao), five apple cultivars (*M. domestica* cv. Fuji, cv. Ohrin, cv. Jonagold, cv. Jonathan, and cv. Mutsu), cherry (*Prunus avium*), Japanese plum (*Prunus salicina*), apricot (*Prunus armeniaca*), Japanese apricot (*Prunus mume*), almond (*Prunus dulcis*), prune (*Prunus domestica*), pear (*Pyrus communis*), Japanese pear (*Pyrus pyrifolia*), strawberry (*Fragaria × ananassa*), raspberry (*Rubus idaeus*), loquat (*Eriobotrya japonica*), quince (*Cydonia oblonga*), juneberry (*Amelanchier* spp.), aloe vera (*Aloe vera*), pineapple (*Ananas comosus*), papaya (*Carica papaya*), orange (*Citrus sinensis*), satsuma orange (*Citrus unshu*), melon (*Cucumis melo*), Japanese persimmon (*Diospyros kaki*), fig (*Ficus carica*), mango (*Mangifera indica*), banana (*Musa acuminata*), avocado (*Persea americana*), blueberry (*Vaccinium corymbosum*), grape (*Vitis* spp.), and kiwifruit (*Actinidia deliciosa*) were purchased from local suppliers. Rice (*Oryza sativa*), soybean (*Glycine max*), maize (*Zea mays*), wheat (*Triticum aestivum*), potato (*Solanum tuberosum*), carrot (*Daucus carota*), onion (*Allium cepa*), Chinese cabbage (*Brassica rapa*), spinach (*Spinacia oleracea*), cucumber (*Cucumis sativus*), and tomato (*Solanum lycopersicum*) were also purchased from local supermarkets. Chinese quince (*Chaenomeles sinensis*) was obtained as a preserve and hawthorn (*Crataegus* spp.) as a dried fruit puree from local supermarkets.

Incurred Food Samples. For the sensitivity study, incurred food samples, strawberry jam and cookies, were prepared by spiking their raw ingredients with peach (*P. persica* cv. Hakuho) and apple (*M. domestica* cv. Fuji). The spiking materials were prepared by freeze-drying freshly peeled fruit and thoroughly mixing with an equal weight of calcium carbonate powder as a dispersant. Total soluble protein concentrations in the spiking materials were determined with the 2-D Quant Protein assay kit (GE Healthcare U.K., Ltd.) after extraction of the proteins with 0.1 M Tris-HCl buffer (pH 7.4, containing 0.5 M NaCl, 0.5% sodium dodecyl sulfate, and 2% β-mercaptoethanol) for 16 h at room temperature. They were 5.7 mg/g for peach and 2.7 mg/g for apple.

The strawberry jam was prepared as follows: Fifty grams of fresh strawberry was homogenized in a food processor to obtain a smooth puree, to which a calculated amount of each spiking material was added and mixed thoroughly. The mixture was cooked over low heat until the total weight was reduced to 65% of the initial weight. After 40 g of sugar was added to the mixture, the pH was adjusted to 3.2 with 0.3 g of citric acid. The mixture was then kept at 80 °C for 30 min.

The cookies were prepared as follows: One hundred grams of wheat flour, 75 g of sugar, 23 g of shortening, 15 g of butter, 1.5 g of salt, 0.12 g of baking soda, 0.37 g of cream of tartar, 25 g of water, and the calculated amount of the spiking materials were thoroughly mixed. The mixed dough was made into disks of approximately 3 cm in diameter and 0.5 cm in thickness and baked at 180 °C for 15 min. The baked cookies were crushed using a mixer mill IFM-650D (Iwatani Corp., Japan) to make a uniform powder. On the basis of the total soluble protein concentrations in the spiking materials as determined with the 2-D Quant Protein assay kit and the final weight of the incurred samples, the total soluble protein concentration of peach and that of apple in the incurred samples were calculated to be 12 μg/g each in the strawberry jam and 10 μg/g each in the cookies.

The final concentrations of slightly higher than 10 μg/g in the strawberry jam were due to evaporative loss of water during preparation. The incurred food samples were stored in a deep freezer at −80 °C until use.

DNA Samples. The DNA samples from soybean seed and maize leaf were extracted and purified with a DNeasy plant Mini Kit (Qiagen GmbH, Hilden, Germany). The DNA samples from the other plant materials (0.1−2.0 g of seeds, leaves, and fruit flesh) and the DNA sample from 2.0 g of incurred cookies were extracted with 20 mL of buffer G2 (Qiagen) supplemented with 20 μL of RNase A (100 mg/mL; Qiagen) and 200 μL of Proteinase K (Qiagen) and purified using Genomic-tip 20/G (Qiagen). The DNA sample from the incurred strawberry jam was prepared in the same way as the incurred cookies except that 0.8 g of autoclaved polyvinylpolypyrrolidone (Sigma-Aldrich Co., St. Louis, MO) was added to 20 mL of the supplemented buffer G2 to avoid possible PCR inhibition by phenolic substances in the sample.[25,26]

The DNA concentration was determined by measuring the absorbance at 260 nm. The DNA samples were diluted to 20 ng/μL with TE (pH 8.0) to make templates for PCR. For the sensitivity studies, DNA samples from a peach and an apple cultivar were serially diluted with a 20 ng/μL salmon testis DNA solution to obtain PCR templates containing 2 fg/μL−2 ng/μL of the target DNA.

The quality of all DNA samples was confirmed by amplifying a fragment of plant chloroplast DNA with the CP 03-5′ (5′-CGG ACG AGA ATA AAG ATA GAG T-3′) and CP 03-3′ (5′-TTT TGG GGA TAG AGG GAC TTG A-3′) primer pairs.[27]

Primers. We designed several candidate primers on various barcode gene regions and picked up the pairs that would achieve the desired sensitivity and specificity.

For peach PCR, the sense primer ppersica-F (5′-TGG TCG TAA TAA AAA GTC AAA A-3′) and the antisense primer ppersica-R (5′-CGT AAA CGC TCT AAT TTT AAT AG-3′) designed on the trnS-trnG IGS region of *P. persica* (GenBank accession no. AY500733) were selected. The second base from the 3′ end of the ppersica-R primer is a deliberate mismatch introduced to improve specificity.[28]

For apple PCR, the sense primer malus-F (5′-ATC ATT GTC GAA CCT GCA CG-3′) and the antisense primer malus-R (5′-ACA CGC GCC GGT GTA A-3′) designed on the ITS-1 region of *M. domestica* cv. Fuji newly sequenced for this work (GenBank accession no. AB636343) were selected.

PCR simulations were performed by using Amplify 1.0 (Bill Engels, Genetics, University of Wisconsin, Madison, WI)[29] to predict whether the designed primer pairs would give PCR products of the target size from the DNA sequences of the trnS-trnG IGS region and ITS-1 region deposited in GenBank.

PCR. PCRs were performed in 0.2 mL reaction tubes in a final volume of 25 μL, containing 1× buffer (PCR buffer II), 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.625 unit of AmpliTaq Gold (Applied Biosystems, Foster City, CA), each primer pair (peach PCR, 0.5 μM each of ppersica-F and ppersica-R primer; apple PCR, 0.4 μM each of malus-F and malus-R primer), and 5 fg−50 ng of template DNA. Amplification was performed in a GeneAmp PCR System 9700 (9600 emulation mode, i.e., ramping speed of 1 °C/s) or 9600 (Applied Biosystems) by using the following conditions: for peach PCR, preincubation at 95 °C for 10 min, 45 cycles consisting of denaturation at 95 °C for 30 s and annealing/extension at 58 °C for 1 min, and a final extension at 72 °C for 7 min; for apple PCR, preincubation at 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 1 min, and a final extension

at 72 °C for 7 min. The PCR products (7.5 μL) were electrophoresed on a 3% agarose gel containing ethidium bromide and analyzed with a ChemiDoc XRS illuminator (Bio-Rad Laboratories, Inc., Hercules, CA). The PCR with the CP 03-5′/CP 03-3′ primer pair[27] yielded the product of the expected size from all DNA samples. The DNA sequences of the PCR products from peach and apple were determined by direct sequencing with a BigDye Terminator v1.1 Cycle Sequencing Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

■ RESULTS

PCR Simulation. The specificity of the designed primer pairs was assessed by performing PCR simulations. For peach PCR, a total of 165 sequences of 125 species obtained from GenBank were subjected to the simulation. As shown in Table 1, with the ppersica-F and -R primer pair, the PCR product of the target size (74 bp) was predicted only from one sequence each of the trnS-trnG IGS region of *P. persica* and *P. mira*, but not from the other 163 sequences of 123 nontarget species, which include 50 *Prunus* spp., 59 Rosaceae species other than *Prunus*, and the other 14 plant foods.

The lower-than-maximum weight number of 4 predicted for the target *P. persica* was due to the deliberate mismatch introduced at the second base from the 3′ end of the reverse primer, ppersica-R, for improvement of the specificity.

For apple PCR, a total of 142 sequences of 125 species were subjected to the simulation. As shown in Table 2, with the malus-F and -R primer pair, the PCR product of the target size (134 bp) was predicted only from a total of 15 ITS-1 sequences of 6 *Malus* spp., that is, 10 sequences of *M. domestica* and one sequence each of 5 closely related *Malus* spp. (*M. asiatica, M. niedzwetzkyana, M. prunifolia, M. sieversii,* and *M. sylvestris*). The PCR product of the target size was not predicted from the 22 sequences of more distant 22 *Malus* spp. and the remaining 105 sequences of 104 nontarget species, which include 47 Maloideae species other than *Malus*, 9 Rosaceae species other than Maloideae, and the other 48 plant foods.

The lower-than-maximum weight number of 5 predicted for the target *M. domestica* sequences previously registered in GenBank was due to the fact that those sequences began only at the position corresponding to the eighth base from the 5′ end of the malus-F primer, which was designed on the new sequence (AB636343).

PCR Experiments. Specificity and sensitivity of the developed methods were confirmed by PCR experiments. As shown in Figure 1, the peach PCR method amplified a product of the target size (74 bp) from 50 fg of DNA extracted from three cultivars of peach (*P. persica* cv. Hakuho, cv. Kawanakajima hakuto, and cv. Golden peach), two cultivars of nectarine (*P. persica* var. *nucipersica* cv. Flavortop and cv. Shuho), and one cultivar of flat peach (*P. persica* var. *platycarpa* cv. Da Hong Pan Tao). However, as shown in Figure 2, it did not amplify such a product from 50 ng of DNA extracted from the other 36 fruits and vegetables tested, including 11 nontarget Rosaceae fruits. The nucleotide sequence analysis of the PCR product from *P. persica* cv. Hakuho confirmed that the target sequence of peach had been amplified (data not shown). A nonspecific product of >1500 bp in size was observed from spinach (Figure 2, lane 34), which was easily distinguishable from the target 74 bp product.

As shown in Figure 3, the apple PCR method amplified a product of the target size (134 bp) from 500 fg of apple DNA extracted from five cultivars (*M. domestica* cv. Fuji, cv. Ohrin, cv. Jonagold, cv. Jonathan, and cv. Mutsu). However, as shown

Table 1. Summary of Peach PCR Simulation Results

scientific name	common name	GenBank accession no.	weight no.[a]
125 species in total		165 sequences in total	
52 *Prunus* spp.		61 sequences in total	
Prunus persica	peach	AY500733	4
Prunus mira	smoothpit peach	AY500732	4
Prunus armeniaca	apricot[b]	AY500725	—
Prunus avium	cherry[b]	AY871252	—
Prunus domestica	prune[b]	AY500719	—
Prunus dulcis	almond[b]	AY500730	—
Prunus mume	Japanese apricot[b]	AY500726	—
Prunus salicina	Japanese plum[b]	AY500722	—
44 other *Prunus* spp.		53 sequences	—
59 Rosaceae species other than *Prunus*		90 sequences in total	
Malus domestica	apple[b]	AY461515 + 7 sequences	—
39 *Crataegus* spp.	hawthorn[b]	EF127091 + 53 sequences	—
Pyrus pyrifolia	Japanese pear[b]	AB545981	—
Amelanchier arborea	juneberry[b]	EF127115	—
Fragaria × ananassa	strawberry[b]	FJ422327	—
16 other Rosaceae species other than *Prunus*		25 sequences	—
14 plant foods other than the above		14 sequences in total	
Vaccinium myrtillus	blueberry[b]	DQ073200	—
Daucus carota	carrot[b]	NC_008325	—
Cucumis sativus	cucumber[b]	NC_007144	—
Solanum melongena	egg plant	AY555465	—
Vitis vinifera	grape[b]	NC_007957	—
Lactuca sativa	lettuce	NC_007578	—
Zea mays	maize[b]	NC_001666	—
Citrus sinensis	orange[b]	NC_008334	—
Solanum tuberosum	potato[b]	NC_008096	—
Oryza sativa (japonica)	rice[b]	X15901	—
Glycine max	soybean[b]	NC_007942	—
Spinacia oleracea	spinach[b]	NC_002202	—
Solanum lycopersicum	tomato[b]	NC_007898	—
Triticum aestivum	wheat[b]	AB042240	—

[a]An approximate indicator (ranging from 1 to 6) of the quality of matches and the strength of amplification predicted. The larger the weight number, the higher the probability of amplification. — indicates no PCR product of the target size was predicted. [b]Absence of amplification product of the target size was confirmed through PCR experiments as well.

in Figure 4, it did not amplify such a product from 50 ng of DNA extracted from the other 41 fruits and vegetables tested, including 16 nontarget Rosaceae fruits. The nucleotide sequence analysis of the PCR product obtained from *M. domestica* cv. Ohrin confirmed that the target sequence of apple had been amplified (data not shown). A nonspecific product of about 250 bp from pear (Figure 4, lane 10) and another of >1500 bp from spinach (Figure 4, lane 39) were observed, but were easily distinguishable from the target 134 bp product.

Table 2. Summary of Apple PCR Simulation Results

scientific name	common name	GenBank accession no.	weight no.[a]	scientific name	common name	GenBank accession no.	weight no.[a]
125 species in total		142 sequences in total		*Prunus mume*	Japanese apricot[b]	AF318728	−
28 *Malus* spp.		37 sequences in total		*Prunus salicina*	Japanese plum[b]	AF318725	−
Malus domestica	apple	AB636343	6	*Prunus persica*	peach[b]	AF318741	−
Malus domestica	apple	U16195 + 8 sequences	5	*Prunus domestica*	prune[b]	AF318713	−
Malus asiatica		AF186494	5	*Rubus idaeus*	raspberry[b]	AF055757	−
Malus niedzwetzkyana	Niedzvetzky apple	AF186497	4	*Fragaria × ananassa*	strawberry[b]	AF163494	−
Malus prunifolia	plumleaf crab apple	AF186500	5	48 plant foods other than the above		48 sequences in total	
Malus sieversii	Asian wild apple	AF186493	5	*Aloe vera*	aloe vera[b]	AF234345	−
Malus sylvestris	European wild apple	FJ899096	5	*Persea americana*	avocado[b]	AF272322	−
22 other *Malus* spp.		22 sequences	−	*Vaccinium myrtillus*	blueberry[b]	AF382732	−
47 Maloideae species other than *Malus*		48 sequences in total		*Daucus carota*	carrot[b]	AY552527	-
Crataegus mollis	hawthorn[b]	U16190	−	*Brassica rapa*	Chinese cabbage[b]	AF128097	−
Pyrus pyrifolia	Japanese pear[b]	AF287240	−	*Cucumis sativus*	cucumber[b]	AY833602	−
24 *Amelanchier* spp.	juneberry[b]	U83922 + 24 sequences	−	*Ficus tonduzii*	fig[b]	AY730140	−
Eriobotrya japonica	loquat[b]	U16192	−	*Vitis vinifera*	grape[b]	AF365988	−
Cydonia oblonga	quince[b]	AF186531	−	*Actinidia deliciosa*	kiwifruit[b]	AF323830	−
19 other Maloideae species other than *Malus*		19 sequences	−	*Zea mays*	maize[b]	DQ683016	−
9 Rosaceae species other than Maloideae		9 sequences in total		*Mangifera indica*	mango[b]	AB071674	−
Prunus dulcis	almond[b]	AF318754	−	*Cucumis melo*	melon[b]	AJ488233	−
Prunus armeniaca	apricot[b]	AF318756	−	*Allium cepa*	onion[b]	AJ411944	−
Prunus avium	cherry[b]	AF318737	−	*Carica papaya*	papaya[b]	AY461547	−
				Solanum tuberosum	potato[b]	AY875827	−
				Oryza sativa (japonica)	rice[b]	AP008225	−
				Glycine max	soybean[b]	AF144654	−
				Spinacia oleracea	spinach[b]	AF062088	−
				Triticum aestivum	wheat[b]	AF521903	−
				29 other plant foods		29 sequences	−

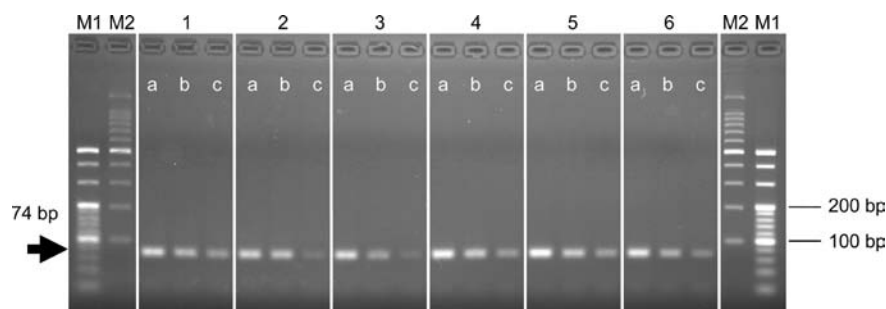[a]See footnotes to Table 1. [b]See footnotes to Table 1.

Figure 1. Amplification of the peach cultivar DNAs by the peach PCR method. The arrowheads indicate the expected size of PCR product. Samples 1−6 are amplification of genomic DNA extracted from *P. persica* cv. Hakuho (1), *P. persica* cv. Kawanakajima hakuto (2), *P. persica* cv. Golden peach (3), *P. persica* var. *nucipersica* cv. Flavortop (4), *P. persica* var. *nucipersica* cv. Shuho (5), and *P. persica* var. *platycarpa* cv. Da Hong Pan Tao (6). Lanes a−c show amplification of 500 fg (a), 50 fg (b), and 5 fg (c) of sample genomic DNA. Lane M1 is a 100 bp DNA ladder DNA marker (Takara Bio Inc.), and lane M2 is 20 bp DNA ladder DNA marker (Takara Bio Inc.).

Analysis of Incurred Food Samples. The sensitivity of the two PCR methods was tested by using the incurred foods containing freeze-dried peach and apple at a level corresponding to their respective total soluble protein concentration of about 10 ppm. As shown in Figure 5, PCR products of the target sizes were detected from 5−50 ng DNA samples extracted from the strawberry jam and the cookies.

■ DISCUSSION

Designing specificity is one of the most important issues in the development of allergen detection methods. A detection method for a particular allergenic food should be able to detect all species that are potentially allergenic to the patients known to be allergic to that particular food, but should not detect species that may not elicit clinical responses in a sizable proportion of the patients so that unnecessary avoidance of foods by those patients may be minimized.

Our primer pairs for peach PCR and apple PCR yielded the target size amplification product from cultivars of peach (including potentially allergenic nectarine and flat peach) and from those of apple, respectively. The absence of the target amplification product from nontarget fruits and vegetables was
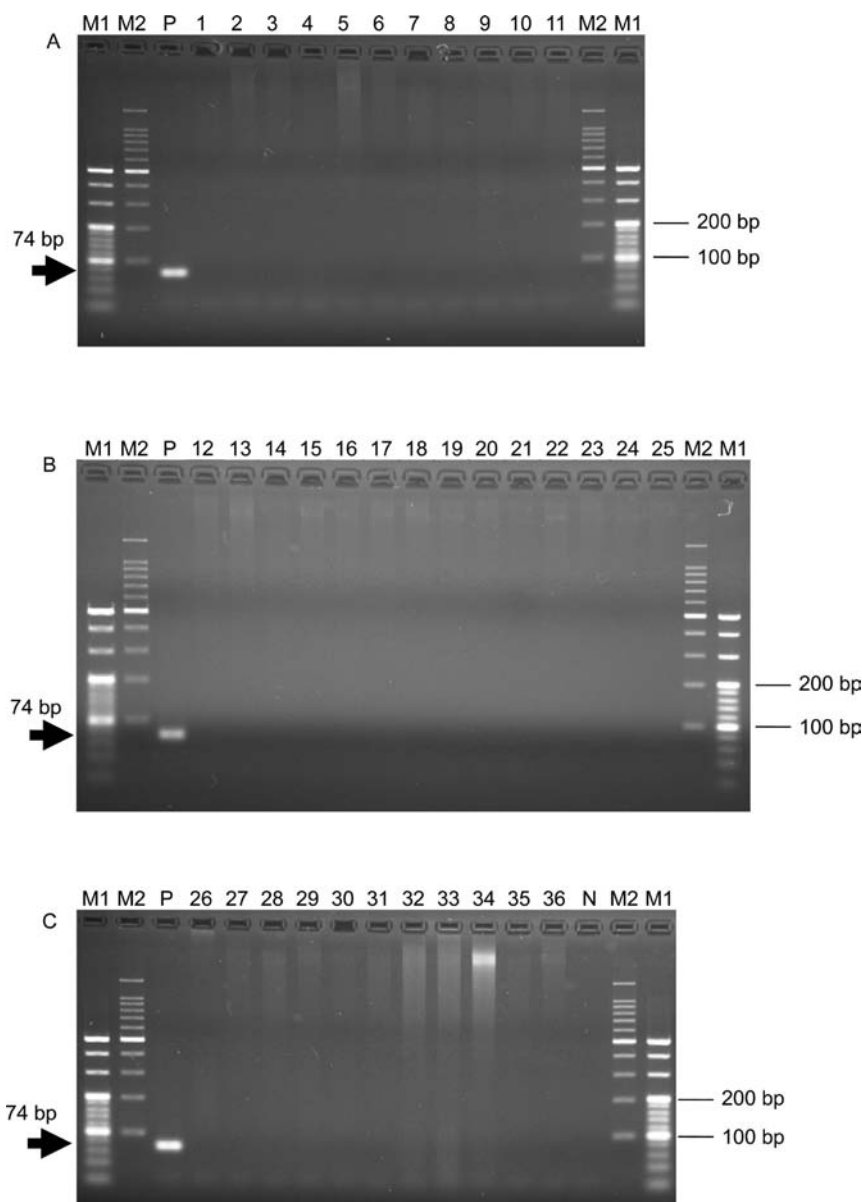
Figure 2. Amplification of the plant DNAs by the peach PCR method: amplification from genomic DNAs of various Rosaceae fruits (A), other fruits (B), and other plant foods (C). The arrowheads indicate the expected size of PCR product. Lane M1 is a 100 bp DNA ladder DNA marker (Takara Bio Inc.), and lane M2 is a 20 bp DNA ladder DNA marker (Takara Bio Inc.). Lanes: P, amplification of 500 fg of genomic DNA extracted from *P. persica* cv. Hakuho; 1−36, amplification of 50 ng of genomic DNA extracted from Japanese plum (1), apricot (2), cherry (3), Japanese apricot (4), almond (5), prune (6), apple cv. Fuji (7), pear (8), Japanese pear (9), strawberry (10), raspberry (11), aloe vera (12), pineapple (13), papaya (14), orange (15), satsuma orange (16), melon (17), Japanese persimmon (18), fig (19), mango (20), banana (21), avocado (22), blueberry (23), grape (24), kiwifruit (25), rice (26), soybean (27), maize (28), wheat (29), potato (30), carrot (31), onion (32), Chinese cabbage (33), spinach (34), cucumber (35), and tomato (36); N, negative control (no template).

also confirmed through PCR simulations and experiments, but with some exceptions. Amplification products of the target sizes were predicted from *P. mira* in the peach PCR simulation and from *M. sieversii*, *M. sylvestris*, *M. asiatica*, *M. prunifolia*, and *M. niedzwetzkyana* in the apple PCR simulation. Because these wild species were unavailable, PCR experiments were not conducted.

P. mira is one of the phylogenetically closest relative of peach,[30] growing wild in the western Himalayan region. It bears inedible fruit and is used as rootstock for peach and almond.[31] To our knowledge, allergenicity of *P. mira* to patients with peach allergies has not been reported. However, in view of its growing region and limited use, unintentional commingling of

its fruit in food products is considered to be unlikely. If *P. mira* turns out to be nonallergenic, its detection by our method gives rise to false positives. Such incidence, however, is considered to be low, because, again, in view of its growing region and limited use, frequent unintentional commingling of its fruit in food products would be unlikely.

M. sieversii from Central Asia and *M. sylvestris* from eastern Europe are the two most likely progenitors of domesticated apple.[13,14,32,33] In addition, *M. asiatica* in East Asia, *M. niedzwetzkyana* in eastern Europe, and *M. prunifolia* in eastern China[13] are among the species phylogenetically most closely related to *M. domestica*.[14,32] Whereas these species are used mainly as rootstock or ornamental plants, there are places where fruits of
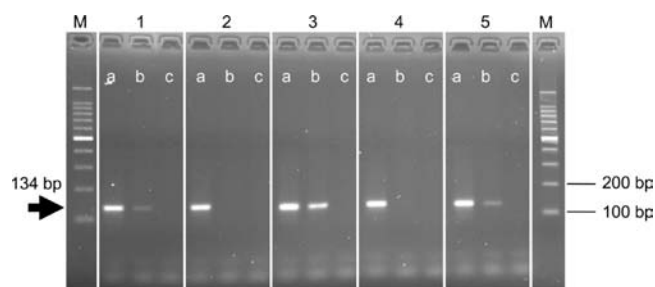
Figure 3. Amplification of the apple cultivar DNAs by the apple PCR method. The arrowheads indicate the expected size of PCR product. Samples 1–5 are amplifications of genomic DNA extracted from *M. domestica* cv. Fuji (1), Ohrin (2), Jonagold (3), Jonathan (4), and Mutsu (5). Lanes a–c represent amplification of 500 fg (a), 50 fg (b), and 5 fg (c) of sample genomic DNA. Lane M is a 100 bp DNA ladder DNA marker (Takara Bio Inc.).

M. asiatica, *M. sieversii*, and *M. sylvestris* are consumed fresh or processed into preserves.[13] Although our search revealed no documented information as to the allergenicity of these wild species, the phylogenetically close relationship with domesticated apple strongly suggests that these species could be allergenic as well. Detection of these wild species by our method may therefore be warranted for the extra safety of apple allergic patients.

The sensitivities of the methods were confirmed by analyzing two incurred foods containing approximately the threshold level (10 ppm) of total soluble proteins from peach and apple. Because amplification products could be obtained from as low as 5 ng, $^1/_{10}$ of the usual amount of DNA in a reaction tube, we considered that the peach and apple detection PCRs were sensitive enough for use as confirmatory tests.
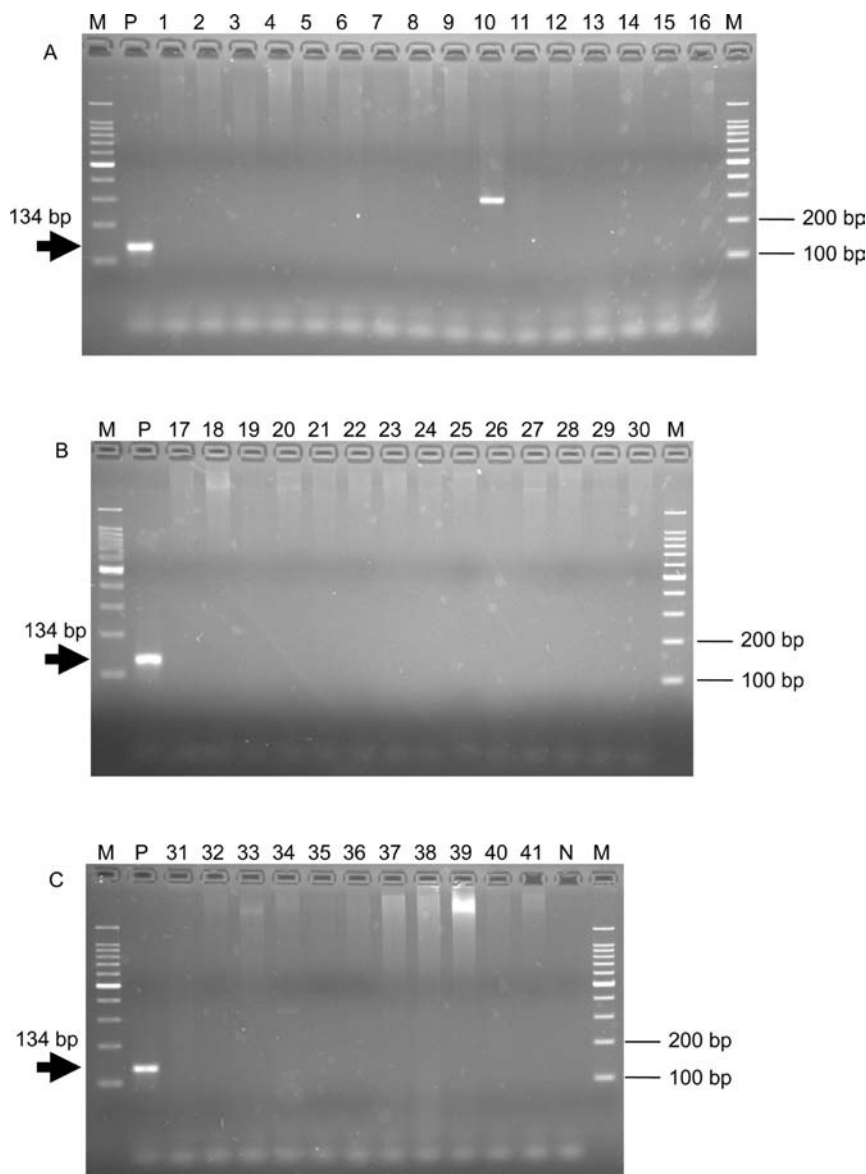

Figure 4. Amplification of the plant DNAs by the apple PCR method: amplification from genomic DNAs of various Rosaceae fruits (A), other fruits (B), and other plant foods (C). The arrowheads indicate the expected size of PCR product. Lane M is a 100 bp DNA ladder DNA marker (Takara Bio Inc.). Lanes: P, amplification of 500 fg of genomic DNA extracted from *M. domestica* cv. Fuji; 1–41, amplification of 50 ng of genomic DNA extracted from strawberry (1), raspberry (2), Japanese plum (3), apricot (4), cherry (5), Japanese apricot (6), almond (7), prune (8), peach cv. Hakuho (9), pear (10), Japanese pear (11), loquat (12), hawthorn (13), juneberry (14), Chinese quince (15), quince (16), aloe vera (17), pineapple (18), papaya (19), orange (20), satsuma orange (21), melon (22), Japanese persimmon (23), fig (24), mango (25), banana (26), avocado (27), blueberry (28), grape (29), kiwifruit (30), rice (31), soybean (32), maize (33), wheat (34), potato (35), carrot (36), onion (37), Chinese cabbage (38), spinach (39), cucumber (40), and tomato (41); N, negative control (no template).
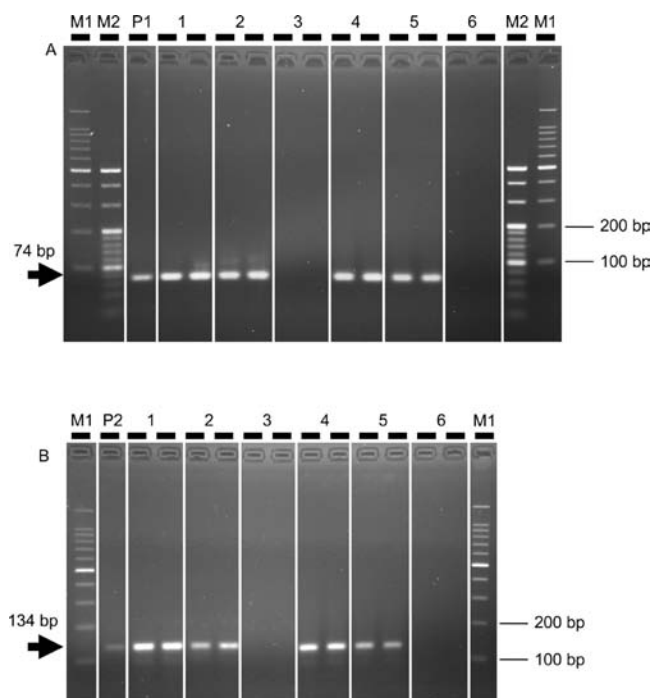
Figure 5. Sensitivity of two PCR methods (A, peach PCR method; B, apple PCR method) determined using incurred foods. The arrowheads indicate the expected size of PCR product. Samples 1–6 are amplifications of model incurred food samples of 50 ng of DNA extracted from incurred jam (1), 5 ng of DNA extracted from incurred jam (2), 50 ng of DNA extracted from control (unspiked) jam (3), 50 ng of DNA extracted from incurred cookies (4), 5 ng of DNA extracted from incurred cookies (5), and 50 ng of DNA extracted from control (unspiked) cookies (6). Lanes: P1, amplification of 50 fg of genomic DNA extracted from Hakuho; P2, amplification of 50 fg of genomic DNA extracted from Fuji. Lane M1 is a 100 bp DNA ladder DNA marker (Takara Bio Inc.), and lane M2 is a 20 bp DNA ladder DNA marker (Takara Bio Inc.).

As Pettersson et al.[28] reported that a mismatch introduced at the second base from the 3′ end of a primer dramatically improved allele specificity in their molecular haplotype determination using allele-specific PCR, we adopted the technique in our primer design for allergen detection PCR. The deliberate mismatch used in the reverse primer for peach PCR (ppersica-R) was found to be effective in improving the specificity of the primer pairs. When it was not used, the primer pair amplified the target size product from nontarget cherry (*P. avium*) in addition to the target *P. persica* (data not shown). Despite the mismatch in the primer, our PCR experiments using incurred foods demonstrated that the target PCR product was amplifiable with sufficient sensitivity. The use of deliberate mismatch was not necessary in the apple PCR primer pair, because the second base from the 3′ end of the reverse primer originally designed on the ITS sequence of *M. domestica* did not match the corresponding base of nontarget *Malus* spp.

Although a proper and accurate label is an effective means of helping allergic patients avoid being exposed to the allergens, the presence of undeclared allergens cannot be ruled out entirely. Therefore, allergen detection methods are indispensable for further controlling the risk of unexpected exposure of patients to allergens. It should be kept in mind that PCR methods target a specific DNA sequence, not allergenic proteins, to detect the presence of an allergenic food. Because DNAs are generally less susceptible to degradation than proteins

are to denaturation and because PCR methods are highly sensitive, they may detect very low levels of a contaminant that may be clinically insignificant. Thus, PCR methods are particularly useful as confirmatory tests after positive results from ELISA determine the levels of contaminating proteins.

In conclusion, we developed two qualitative PCRs, one for peach detection and the other for apple detection. These methods' specificity and sensitivity were considered to be sufficient for the detection of peach or apple contamination at the threshold level for declaration of allergens established by the Japanese food allergen labeling regulation. These methods are expected to be useful for monitoring and controlling possible contamination of foods by peach and apple and, consequently, for preventing unexpected exposure of allergic patients to these fruits.

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Notes
The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; ITS, internal transcribed spacer; IGS, intergenic spacer; rbcL, large subunit of ribulose-1,5-bisphosphate carboxylase; trn (L, S, G, and H), transfer ribonucleic acid (leucine, serine, glycine, and histidine); matK, maturase K; psbA, 32 kDa quinone-binding protein of photosystem II reaction center.

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