Application of the Multiplex PCR Method for Discrimination of *Artemisia iwayomogi* from Other *Artemisia* Herbs

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Received August 7, 2007; accepted January 16, 2008; published online January 18, 2008

Some plants classified in the genus *Artemisia* are used for medicinal purposes. In particular, *A. iwayomogi*, which is referred to as ‘Haninjin,’ is used as an important medicinal material in traditional Korean medicine. However, *A. capillaris*, and both *A. argyi* and *A. princeps*, referred to as ‘Injinho’ and ‘Aeyup,’ respectively, are used for purposes other than those for which ‘Haninjin’ is utilized. However, it is occasionally difficult to differentiate ‘Haninjin’ from ‘Injinho’ and/or ‘Aeyup’ on the basis of their morphological features, particularly when in the dried and/or sliced form. Therefore, the development of a reliable method by which to discriminate ‘Haninjin’ from other *Artemisia* herbs, especially ‘Injinho’ and ‘Aeyup,’ is clearly necessary. We recently determined that the RAPD (random amplified polymorphic DNA) technique can be used to discriminate efficiently between some *Artemisia* herbs. In particular, when applied to RAPD, the non-specific UBC primer 391 (5'-GCG AAC CTC G-3') was demonstrated to amplify PCR products specific to *A. iwayomogi*. Based on the nucleotide sequences of the PCR product, we designed a 2F1 (5'-ACC TCG GAC CTA AAT ACA-3')/ 2F3 (5'-TTA TGA TTC ATG TTC AAT TC-3') primer set to amplify a SCAR (sequence-characterized amplified region) marker of *A. iwayomogi*. Employing this primer set, along with two other primer sets amplifying SCAR markers of ‘Aeyup’ (*A. argyi* and *A. princeps*) and both ‘Injinho’ (*A. capillaris*) and *A. japonica*, which are classified into the same subgroup in a phenogram constructed from RAPD analysis, we developed a multiplex PCR method by which *A. iwayomogi* could be discriminated with certainty from other *Artemisia* herbs. Via this method, we determined not only whether the tested *Artemisia* herb was *A. iwayomogi*, but also which *Artemisia* herbs were tested concurrently with *A. iwayomogi*.

Key words *Artemisia iwayomogi*; *Artemisia* herb; random amplified polymorphic DNA; sequence characterized amplified region marker; multiplex PCR

Artemisia plants, particularly *A. iwayomogi*, *A. capillaris*, *A. princeps*, and *A. argyi*, are important medicinal materials that are utilized in traditional Asian medicines.²⁾ *A. iwayomogi* and *A. capillaris*, referred to in Korea as ‘Haninjin’ and ‘Injinho’, respectively,²⁾ have been utilized traditionally for the treatment of diuresis and as anti-inflammatory agents, as is also the case in China and Japan.^{2,3)} Meanwhile, both *A. princeps* and *A. argyi* are referred to as ‘Aeyup,’ and are employed in the treatment of colic pain, vomiting, and irregular uterine bleeding.^{2,4)}

In traditional Korean medicine markets, a portion of certain specific *Artemisia* herbs tend to be distributed interchangeably with other *Artemisia* herbs.²⁾ For instance, some ‘Haninjin’ is distributed under the name ‘Aeyup.’ However, ‘Aeyup’ may occasionally be substituted with ‘Haninjin’ or ‘Injinho’ in medicinal prescriptions. To address this problem, an efficient method for the identification of *Artemisia* herbs is clearly necessary. Currently, the discrimination of one medicinal herb from another is a process which involves subjective methods, frequently predicated on the morphological features of the plant and/or the observations of a trained individual. However, these methods are not always sufficient for the identification of plants that morphologically resemble each other, particularly when the plants are in a dried and/or sliced state, the common forms of plants when purveyed in an herbal market. Therefore, objective methods, such as those predicated on differences in DNA sequences between organisms,⁵⁾ should be developed. In a previous work,⁶⁾ we

showed that a sequence-characterized amplified region (SCAR) marker⁷⁾ based on the nucleotide sequence of a specific random amplified polymorphic DNA (RAPD) product amplified uniquely from ‘Aeyup’ (both *A. princeps* and *A. argyi*) proved to be a reliable DNA marker for the discrimination of ‘Aeyup’ from other *Artemisia* herbs. As some *Artemisia* herbs can be well-distinguished from each other by RAPD, a SCAR marker of ‘Haninjin’ (*A. iwayomogi*) developed by the same strategy used for the SCAR markers of ‘Aeyup’ might facilitate the discrimination of ‘Haninjin’ from other *Artemisia* herbs, particularly ‘Injinho’ and ‘Aeyup.’ In this work, we developed SCAR markers of *Artemisia* herbs, including ‘Haninjin.’ We then applied these SCAR markers to the development of a method that could be applied not only to identify tested *Artemisia* herbs as ‘Haninjin’ (*A. iwayomogi*), but also to determine which *Artemisia* herbs are being tested concurrently with *A. iwayomogi*.

Using this method, we were able to differentiate ‘Haninjin’ from other *Artemisia* herbs with more certainty as compared with a technique predicated on the amplification of a single DNA marker, such as a SCAR marker. Furthermore, we were able to apply this method to a search for *Artemisia* herbs that are substituted for ‘Haninjin’ and/or mixed with ‘Haninjin.’ In service of these research purposes, we developed a multiplex PCR method⁸⁾ using a primer set to amplify a SCAR marker of ‘Haninjin’, along with two other primer sets to amplify the ‘Injinho’ and ‘Aeyup’ SCAR markers, in order to amplify each SCAR marker of the *Artemisia* herbs in a single

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PCR process.

MATERIALS AND METHODS

Plant Materials Seventeen samples of *Artemisia* herbs were obtained, and are shown in Table 1. These include five samples of *A. iwayomogi*, five samples of *A. capillaris*, three samples of *A. princeps*, two samples of *A. argyi*, and one each of *A. japonica* and *A. keiskeana*.

The Rural Development Administration (RDA) of the Republic of Korea supplied fresh leaves of *Artemisia* herbs, which were acquired within the country. One sample of *A. argyi* was purchased in the Guangxi province of China. The authenticity of the samples was confirmed by the National Institute of Crop Science of the RDA of the Republic of Korea, and the samples were deposited at the Korea Institute of Oriental Medicine (KIOM).

Preparation of Genomic DNA The genomic DNA of each sample was extracted in accordance with the instruction manual for the PureGene DNA purification kit (Gentra, U.S.A.). In order to improve the DNA quality, phenolic compounds and polysaccharides were removed with 10% cetyltrimethyl ammonium bromide (CTAB) and 0.7 M NaCl.

PCR Amplification The PCR for RAPD was conducted in accordance with the method described by Williams *et al.*⁹⁾ using a T-personal cyler (Biometra, Germany). In brief, 600 nM UBC primer (University of British Columbia, Canada), 1 U *Taq* polymerase (ABgene, U.S.A.), and 50 ng of genomic DNA extracted from each sample were utilized for PCR amplification. The PCR process consisted of 36 cycles of the following: pre-denaturation for 5 min at 94 °C, denaturation for 30 s at 94 °C. The annealing process was conducted for 30 s at 37 °C, and the extension process was conducted for 1 min at 72 °C. A final 10 min reaction step was conducted at 72 °C.

To amplify the SCAR markers of *Artemisia* herbs with the designed primer sets, PCR was conducted under identical conditions for RAPD amplification, with the exception of the annealing process conducted at 55 °C.

Multiplex PCR was also conducted using a T-personal cy-

cler (Biometra, Germany). Then, 0.8 pmol of 2F1/2F3 primers, 4 pmol of 354UF3/354UR3 primers, 0.08 pmol of Fb/R7 primers, 2.5 U of *Taq* polymerase (ABgene, U.S.A.), and 10 ng of genomic DNA extracted from each of the *Artemisia* samples were used for amplification. Next, 0.16 pmol of an AYF (5'-ACGGATATCTCGGCTC-3')/AYR (5'-GAACCA-TCGAGTTTTTGAAC-3') primer set, which was designed on the basis of the partial nucleotide sequences of the 5.8 s rDNA of *Arabidopsis*, was utilized to amplify the 95 bp PCR product as an internal standard that could be used to determine the efficiency of PCR amplification. In the multiplex PCR, pre-denaturation was conducted for 12 min at 95 °C. 30 PCR cycles were conducted under the following conditions. Denaturation was conducted for 30 s at 95 °C, annealing was conducted for 30 s at 50 °C, and a final 30-s extension step was conducted at 72 °C. Finally, the PCR reaction was allowed to continue for an additional 7 min at 72 °C. After the amplified products were separated on 1.5% agarose gel, the gel was stained with EtBr (Sigma, U.S.A.). The amplified products were then analyzed using MyImage (Seoulin Biotechnology, Republic of Korea).

Nucleotide Sequencing of PCR Products PCR products separated from the agarose gel were cloned with pGEM T-easy vector (Promega, U.S.A.). Each PCR product was amplified twice *via* independent PCR processes. The nucleotide sequences of the cloned PCR products were determined using two samples isolated from different colonies by Bionex (Republic of Korea).

RESULTS

Artemisia Herbs Are Discriminated through RAPD

Analysis SCAR markers based on the unique RAPD-amplified PCR products of *A. iwayomogi* ('Haninjin') and other *Artemisia* herbs must be developed prior to the development of a multiplex PCR method that can be applied for the discrimination of 'Haninjin' from other *Artemisia* herbs, such as 'Injinho' and 'Aeyup.' Therefore, we initially attempted to ascertain whether *A. iwayomogi* and other *Artemisia* herbs could be discriminated by RAPD. On the basis of the poly-

Table 1

Medicine name	Scientific name	Locality	Nationality	Date of collection	Lane
Aeyup	<i>A. princeps</i>	Bongwha	Korea	2002.9	1
		Jeonju	Korea	2002.5	2
		Uiseong	Korea	2002.9	3
	<i>A. argyi</i>	Suwon	Korea	2002.6	4
		Gvangsih	China	2002.7	5
Injinho	<i>A. capillaris</i>	Jeonju	Korea	2002.5	6
		Jinan	Korea	2002.5	7
		Jinan	Korea	2002.5	8
		Ullungdo	Korea	2002.7	9
		Bonghwa	Korea	2002.9	10
Haninjin	<i>A. iwayomogi</i>	Jecheon	Korea	2002.7	11
		Uiseong	Korea	2002.9	12
		Jinan	Korea	2002.5	13
		Jinan	Korea	2002.5	14
		Bonghwa	Korea	2002.9	15
Others	<i>A. japonica</i>	Suwon	Korea	2002.6	16
	<i>A. keiskeana</i>	Uiseong	Korea	2002.9	17

morphisms revealed by 14 non-specific UBC primers, we assessed the phylogenetic similarity among the *Artemisia* herbs listed in Table 1. We also conducted a phenogram by conducting UPGMA (unweighted pair-group method with arithmetic average) using the NTSYS (numerical taxonomy and multi-analysis system) program. The phenogram constructed in this work (data not shown) was almost identical to a previous phenogram predicated on 11 non-specific UBC primers revealing polymorphisms among *Artemisia* herbs.⁶⁾ In both phenograms, all five of the *A. iwayomogi* samples were categorized in one group, whereas the other *Artemisia* herbs were allocated to the other group. In the other group, samples of both *A. princeps* and *A. argyi* ('Aeyup') were classified into one subgroup, and samples of both *A. capillaris* and *A. japonica* were allocated into the other subgroup. However, the position of *A. keiskeana*, which was clearly separated from other *Artemisia* herbs, differed substantially in the two phenograms. Whereas, in a new phenogram, *A. keiskeana* was positioned on the other group including 'Aeyup' and both *A. capillaris* and *A. japonica* as a separated subgroup, in a previous study, *A. keiskeana* was positioned on one main branch of the phenogram that was separated from the other main branch, which included all samples of *Artemisia* herbs with the exception of *A. keiskeana*.

These results suggested that PCR product(s) amplified uniquely from *A. iwayomogi* and other *Artemisia* herbs might indeed be applicable to the discrimination of *A. iwayomogi* from other *Artemisia* constituents.

Development of a SCAR Marker of *A. iwayomogi* Among the non-specific primers revealing polymorphisms among the *Artemisia* herbs, primer 391 (5'-GCG AAC CTC G-3') generated 700–720 bp PCR products from all of the *A. iwayomogi* samples (Fig. 1). While the RAPD method is sensitive to PCR conditions, the reproducibility of RAPD results tends to be relatively low. In order to deal with this problem, sequence-characterized amplified region (SCAR) markers were first suggested by Paran and Micheltore.⁷⁾ Therefore, based on the nucleotide sequences of these products, we attempted to develop a SCAR marker for *A. iwayomogi*. For the determination of the nucleotide sequences of these PCR products, we sub-cloned each PCR product amplified from each sample of *A. iwayomogi* (Lanes 11–15 in Table 1). According to the determined nucleotide sequences of these PCR products, 707 bp, 714 bp, 718 bp, and 719 bp PCR products were amplified and sub-cloned from lanes 11 and 15, 14, 13, and 12, respectively. The nucleotide sequences of the 707, 714, 718, and 719 bp PCR products were registered as AY866423, AY866420, AY866422, and AY866421, respectively, in the GenBank database at the National Center for Biotechnology Information (NCBI). The similarities of the nucleotide sequences among three of these products, excluding the 707 bp PCR product, were all in excess of 97%. When the 707 bp regions in the nucleotide sequences of each of the three PCR products were compared with that of the 707 bp PCR product, similarities of 88 to 98% were noted. Based on the nucleotide sequences in which the similarities among PCR products were high, some primer sets were designed to amplify the SCAR marker of *A. iwayomogi*. Among them, 2F1 (5'-ACC TCG GAC CTA AAT ACA-3')/2F3 (5'-TTA TGA TTC ATG TTC AAT TC-3') was shown to amplify both the 364 and 365 bp PCR prod-

Fig. 1. Polymorphism among *Artemisia* Herbs Detected by Non-specific UBC Primer 391

Lanes 1–17, *Artemisia* herbs listed in Table 1. M, 100 bp ladder.

ucts, which appeared as a single band on the gel from *A. iwayomogi* (Fig. 3). Among them, the 2F1 (5'-ACC TCG GAC CTA AAT ACA-3')/2F3 (5'-TTA TGA TTC ATG TTC AAT TC-3') primer set designed to concurrently amplify both the 364 and 365 bp SCAR markers (Fig. 2) was also shown to amplify the PCR product, which appeared as a single band on the gel from all of the *A. iwayomogi* samples (Fig. 3). Using capillary electrophoresis, we confirmed that these two predicted SCAR markers were concurrently amplified from all *A. iwayomogi* samples (data not shown). Meanwhile, no PCR products were generated by this primer set in other *Artemisia* herbs. According to these results, the 2F1/2F3 primer set was capable of amplifying the SCAR marker that distinguished *A. iwayomogi* from other *Artemisia* herbs.

Development of a Common SCAR Marker of Both *A. capillaris* and *A. japonica* As previously mentioned, 'Injinho' (*A. capillaris*) is one of the *Artemisia* herbs that must be discriminated clearly from *A. iwayomogi*. Therefore, we also attempted to develop a SCAR marker for *A. capillaris*. However, as shown in a previous study,⁶⁾ both *A. capillaris* ('Injinho') and *A. japonica* are classified into Subgroup 1A of the phenogram. Because of the phylogenetic similarity between the two *Artemisia* herbs, we attempted to develop a common SCAR marker of both *A. capillaris* ('Injinho') and *A. japonica*. In the previous study,⁶⁾ we determined that the non-specific UBC primer 354 (5'-CTA GAG GCC G-3') specifically generated a PCR product of approximately 600 bp from both *A. capillaris* and *A. japonica*. In this study, we ascertained the nucleotide sequences of these 600 bp PCR products from both *A. capillaris* and *A. japonica* (Fig. 4). These nucleotide sequences have been registered in the NCBI GenBank database as EF204532 and ER893952, respectively. We noted an 87% similarity between the sequences of the PCR products from *A. capillaris* and *A. japonica*. Based on the established nucleotide sequences, we designed primer sets to concurrently amplify a SCAR marker common to both *A. capillaris* and *A. japonica*. Finally, we selected one primer set, 354UF3 (5'-CTA GAG GCC GAC GCG GAC-3')/354UR3 (5'-ATG CTT TTG GCT ATA TGC AGT C-3'), in order to amplify a 396 bp SCAR marker common to both *A. capillaris* and *A. japonica* (Fig. 5).

Development of a Multiplex PCR Method for the Discrimination of *A. iwayomogi* from Other *Artemisia* Herbs and for the Determination of Which *Artemisia* Herbs Are Being Tested In the previous study,⁶⁾ we developed an Fb/R7 primer set that amplified a 254 bp SCAR marker common to both *A. princeps* and *A. argyi* ('Aeyup'). Along with

Fig. 2. Comparison of the Nucleotide Sequences of PCR Products of *A. iwayomogi* Amplified Using UBC Primer 391

Bold boxes around nucleotides indicate a designed primer set. Nucleotides with a highlighted background are matching sequences of UBC primer 391.

Fig. 3. PCR Products of the Designed Primer Set (2F1/2F3) from 17 Kinds of *Artemisia* Herbs

Lanes 1—17, *Artemisia* herbs listed in Table 1. M, 100 bp ladder.

this primer set, the 2F1/2F3 and 354UF3/354UR3 primer sets were applied to the development of a multiplex PCR method that could be used to discriminate ‘Haninjin’ (*A. iwayomogi*) from some other *Artemisia* herbs, and to concurrently determine which *Artemisia* herbs were tested along with *A. iwayomogi*. As can be seen in Fig. 6, while both the 364 and 365 bp PCR products appeared solely in ‘Haninjin’ (*A. iwayomogi*), the 254 bp and 394 bp PCR products were generated in the ‘Aeyup’ and ‘Injinho’ samples. As had been expected, the 354UF3/354UR3 primer sets also yielded a 394

bp PCR product in *A. japonica*. Meanwhile, no amplification products were generated in *A. keiskeana*, a member of Sub-group IB, after multiplex PCR. Therefore, this multiplex PCR method might be applicable to the discrimination of *A. iwayomogi* from other *Artemisia* herbs. Furthermore, this method could also be used to determine which *Artemisia* herbs are present in a sample, or at least into which sub-group(s) the *Artemisia* herbs tested along with *A. iwayomogi* should be classified.

DISCUSSION

For centuries, ‘Haninjin’ (*A. iwayomogi*) has been used as a traditional medicine in Korea.^{3,10} In particular, aqueous ‘Haninjin’ extract is frequently prescribed as a crude drug for the treatment of a variety of liver diseases.¹¹ Recently, other pharmacological effects of *A. iwayomogi* have been observed. *A. iwayomogi* extract has been shown to inhibit immediate-type allergic reactions and inflammatory cytokine secretion.¹² Furthermore, a carbohydrate fraction purified from a crude water-soluble *A. iwayomogi* extract was demonstrated to suppress artificially-induced mouse thymocyte apoptosis *via* the modulation of *Fas* gene expression, which plays important roles in the damage induced by dioxin, as well as in some diseases.¹³ While both ‘Haninjin’ (*A. iwa-*

Fig. 4. Comparison of the Nucleotide Sequences of PCR Products of *A. capillaris* and *A. japonica* Amplified Using UBC Primer 354

Bold boxes around nucleotides indicate a designed primer set. Nucleotides with a highlighted background are matching sequences of UBC primer 354.

Fig. 5. PCR Products of the Designed Primer Set (354UF3/354UR3) from 17 Kinds of *Artemisia* Herbs

Lanes 1—17, *Artemisia* herbs listed in Table 1. M, 100 bp ladder.

Fig. 6. Multiplex PCR Products Using Three Primer Sets (2F1/2F3, 354UF3/354UR3, Fb/R7) from 17 Kinds of *Artemisia* Herbs

Lanes 1—17, *Artemisia* herbs listed in Table 1. M, 100 bp ladder.

yomogi) and 'Injinho' (*A. capillaris*) are often prescribed for specific diseases or symptoms, 'Haninjin' is strictly differentiated from 'Injinho' in the Republic of Korea.²⁾ Occasionally, 'Haninjin' and 'Injinho' are used in lieu of 'Aeyup' for medicinal prescriptions. Therefore, in this study, we have attempted to develop objective methods predicated on the relevant genetic information in order to discriminate 'Haninjin' from other *Artemisia* herbs, particularly 'Injinho' (*A. capillaris*) and 'Aeyup' (*A. princeps* and *A. argyi*). In service of this objective, we attempted to devise a multiplex PCR method as a more certain and useful discrimination method on the basis of the nucleotide sequences of some polymorphic PCR products of the *Artemisia* herbs after RAPD.

A prerequisite for this approach is that the *A. iwayomogi* samples must first be classified into the same group or subgroup in the phenogram of *Artemisia* herbs constructed on the basis of the RAPD results. In addition, other *Artemisia* samples must be classified into other group(s) or subgroup(s) into which *A. iwayomogi* samples have yet to be classified. In the previous study,⁶⁾ a phenogram constructed on the basis of polymorphisms revealed through 11 primers was employed as the basis for the development of a SCAR marker of 'Aeyup' (both *A. princeps* and *A. argyi*). Because the reproducibility of RAPD is somewhat dubious, we constructed a

new phenogram predicated on the polymorphisms revealed by 14 non-specific UBC primers, including primers 303 (5'-GCG GGA GAC C-3'), 321 (5'-ATC TAG GGA C-3'), and 323 (5'-GAC ATC TCG C-3'), in order to confirm the separation of *Artemisia* herbs on the phenogram. According to a new phenogram constructed in this study, all 'Haninjin' samples were classified into the same group. In contrast, samples of 'Injinho' (*A. capillaris*) and 'Aeyup' are classified into other groups. Furthermore, these two *Artemisia* herbs are classified into different subgroups. As shown in the new phenogram, *A. japonica* is classified into a subgroup that includes *A. capillaris*. This phylogenetic similarity between *A. capillaris* and *A. japonica* was also noted in the previous study.⁶⁾ Therefore, the phenogram became the basis of the development of SCAR markers for *A. iwayomogi*, and for both *A. capillaris* and *A. japonica*. Furthermore, this phenogram was applied for the development of a multiple PCR method.

Four PCR products from *A. iwayomogi* were simultaneously amplified using primer 391 (Fig. 2), and one PCR product was amplified from both *A. capillaris* and *A. japonica* using primer 354 (Fig. 4). The nucleotide sequences of these PCR products were used to design primer sets for the amplification of each of the SCAR markers. These nu-

cleotide sequences were compared with the sequences in the NCBI database in order to ascertain whether or not the PCR products harbored certain ORFs (open-reading frames). 230–250 bp fragments of the 707 bp PCR product were found to bear a similarity of 60% with the cDNAs cloned from *Oryza sativa* (CI319338) and *Brassica napus* (CD841471), the functions of which have yet to be elucidated. In addition, 220–415 bp fragments of the 714, 718, and 719 bp PCR products evidenced 50–60% similarity with the cDNAs cloned from *Antirrhinum majus* (AJ808326) and *Chlamydomonas reinhardtii* (BG854434), the functions of which also have yet to be determined. However, the 270 bp fragment of these three PCR products evidences 55–60% similarity with the lipase class 3 family protein of *Arabidopsis* (AT4G00500). As mentioned in the results section, both 364 bp and 365 bp SCAR markers of *A. iwayomogi* based on the nucleotide sequences of 707 bp, and three of 714, 718 and 719 bp PCR products respectively (Fig. 2), were concurrently amplified in all five samples of *A. iwayomogi* (data not shown). On the basis of these results, we speculate that these four PCR products amplified by primer 391 from five *A. iwayomogi* samples could harbor at least partial ORFs of the genes, which are members of a specific multi-gene family.

From *A. capillaris* and *A. japonica*, which were classified into the same subgroup on the phenogram (data not shown), a 599 bp PCR product was amplified by primer 354. We noted an 87% similarity between the nucleotide sequences of two *Artemisia* herbs, as shown in Fig. 4. As this work was principally focused on the discrimination of *A. iwayomogi*, we have not attempted to further discriminate *A. capillaris* from *A. japonica* using DNA markers. The nucleotide sequence of the PCR product amplified by primer 354 from *A. capillaris* was compared to the sequences deposited in the NCBI database. The 211 bp fragment of the PCR product evidenced a similarity of 63% with the cDNA; these findings were similar to those from the electron transport complex protein, *rnfD*, which was cloned in *Eleusine coracana* (EB637139). In addition, the 422 bp fragment of this PCR product evidenced 57% similarity with a cDNA found in a navel orange (*Citrus sinensis*) callus, the function of which remains unknown (CV713837).

Therefore, the nucleotide sequences used to design the primer could also harbor partial protein information. According to a laboratory manual,¹⁴⁾ there is a high theoretical probability that an 18-mer primer will anneal at only one position in the majority of eukaryotic genomes, which are composed of approximately 10^9 – 10^{10} bp. Based on this information, we designed ten primer sets ranging from 18-mer to 22-mer in order to amplify the SCAR markers applied for the discrimination of *Artemisia* herbs. Among them, the pair of the 18-mer, 2F1, and the 20-mer, 2F3, concurrently amplified SCAR markers of *A. iwayomogi*, with sizes of 364 and 365 bp. In Fig. 3, these two PCR products are shown as a single band on the gel. Meanwhile, for the common SCAR marker of both *A. capillaris* and *A. japonica*, a primer set

(19-mer 354UF3 and 22-mer 354UR3) that amplified a 394 bp product (Fig. 5) was screened among a combination of 18 primers, which ranged from 19-mer to 23-mer. Along with these two primer sets, we also developed a multiplex PCR method that could be applied to distinguish ‘Haninjin’ from other *Artemisia* herbs, particularly ‘Injinho’ and ‘Aeyup’. In the multiplex PCR technique, the template DNA isolated from *Artemisia* was conserved significantly as compared to that utilized in conventional PCR. Furthermore, the process was also more efficient in terms of the time, chemicals, and enzymes required for the running of PCR to amplify each SCAR marker of the *Artemisia* herbs.^{8,15)} As is shown in Fig. 6, under the multiplex PCR conditions developed in this study, a 364–365 bp SCAR marker of *A. iwayomogi* was amplified, and was determined to be located exactly between the 254 bp and 394 bp bands amplified from both *A. princeps* and *A. argyi*, as well as both *A. capillaris* and *A. japonica*. These results suggested that the developed multiplex PCR method could indeed be utilized for the accurate discrimination of *A. iwayomogi*. Furthermore, this technique was appropriate for the determination of *Artemisia* herbs tested along with *A. iwayomogi* could be classified, or at least to which subgroups the tested *Artemisia* herbs could be classified.

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