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Original Research Article

Molecular Identification of *Reynoutria japonica* Houtt. and *R. sachalinensis* (F. Schmidt) Nakai Using SNP Sites

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Abstract - *Reynoutria japonica* and *R. sachalinensis* have been used as medicinal resources in Korea. However, it is difficult to identify and determine these medicinal herbs correctly because they are usually customized and purchased as the fragmented rhizomes types. To develop molecular markers for distinguishing two species, we analyzed and compared the chloroplast DNA sequences of seven loci (*atpB*, *matK*, *ccD-psaI*, *atpF-H*, *trnL-trnF*, *psbK-I* and *rpl32-trnL*). Among them, we found two effective SNPs in *psbK-I* region for *R. japonica* and *atpF-H* region for *R. sachalinensis*. Based on these SNP sites, we designed the new *R. japonica*- specific primer which is able to amplify 300 bp fragment in *psbK-I* region. A similar strategy was applied for the *atpF-H* region of *R. sachalinensis*. These molecular markers would be successfully applied to recognize *R. japonica* and *R. sachalinensis*.

Key words - Molecular identification, *Reynoutria japonica*, *Reynoutria sachalinensis*, SNP

Introduction

Molecular marker usually have several advantages such as reflecting genetic property of species as well as variety, being influenced by any surroundings and being easily processed, stored and shared information. According to the technique, there are five kinds of molecular markers; Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR) and Single Nucleotide Polymorphism (SNP). Especially, SNP molecular marker can be used easily as well as highly reproducibility and formation of polymorphism because of using technique based on PCR. Therefore, there are many studies of genetic polymorphisms of angiosperms and animals and analysis relationship of species as well as variety (Batley *et al.*, 2002; Deynze *et al.*, 2007; Kim and Misra, 2007). For examples, Korean Ginseng (*Panax ginseng*)-specific primer was developed based on SNP in ITS region (In *et al.*, 2010). And, Kim *et al.* (2012) used *rbcL* and ITS regions to devise the specific primer for identification of *Schisandra chinensis* with other related species.

Amplification Refractory Mutation System (ARMS) has been usefully applied with SNP as a molecular genetic technique (Kim *et al.*, 2012). For ARMS, when the target DNA does not match the base in 3' end of primer, there is no extension step by DNA polymerase in PCR progress. Then, it is possible to develop the primers based on ARMS-PCR for the species particularity molecular markers. The SNP markers are helpful for the more effectively conformation of identification and origin of medicine herbs.

Polygonaceae Juss. are monophyletic and sister group of Plumbaginaceae Juss. (Chase *et al.*, 1993; Fay *et al.*, 1997; Chase *et al.*, 2002). However, the classification of

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species within this family remains unclear because of morphological variability. Schuster *et al.* (2011) used five chloroplast regions and two nuclear genes to reconstruct the relationship within Polygonaceae. Their results showed the clear relationship of *Fallopia* Adans. and *Reynoutria* Houtt. which were merged and separated several times during the complex taxonomic history of Polygonaceae. In addition, the recognition of *Fallopia* as sister to *Reynoutria* was supported by the different chromosome base numbers which were x=10 for the former (Jaretzky, 1928) and x=11 for the latter (Bailey and Stace, 1992).

In Polygonaceae, *Reynoutria* comprises of seven species located in temperate region of the Northern Hemisphere including East-Asia, Europe and North America (Conolly, 1977; Hollingsworth *et al.*, 1999; Bailey *et al.*, 2007; Gammon and Kesseli, 2010; Stoll *et al.*, 2012; Dorigo *et al.*, 2012). Five species of *Reynoutria*; including *R. japonica* Houtt. (*Fallopia japonica=Polygonum cuspidatum*; Ho-jang-geun), *R. sachalinensis* (F. Schmidt) Nakai (*F. sachalinensis=P. sachalinensis*; Wang-ho-jang-geun), *R. forbesii* (Hance) T. Yamaz. (*F. forbesii*=P. *forbesii*; Gam-jeol-dae), *R. ciliinervis* (Nakai) Moldenke (*F. ciliinervis=P. ciliinerve*; Na-do-ha-su-o) and *R. multiflora* (Thunb.) Moldenke (*F. multiflora=P. multiflorum*; Ha-su-o) are distributed in Korea (Baik *et al.*, 1986; Lee *et al.*, 1997; Kim and Park, 2000; Do *et al.*, 2011). Especially, *R. sachalinensis* can be only found in Ulleung-do island and Dok-do island in Korea. And *R. japonica* and *R. sachalinensis* still have taxonomic confusion and are difficult of determining species boundaries (Steward, 1930; Park *et al.*, 2011) because of the complex patterns of variation in morphological characters which are observed according to the habitat and environment as like other Polygonaceae species (Bailey and Stace, 1992). Moreover, those species are highly variable in morphology and chromosome numbers, resulting in notable controversy about differentiation degree, validity of classification, setting of limitation and order (Steward, 1930; Ohwi, 1984; Kim and Park, 2000, Park *et al.*, 2011).

R. japonica and *R. sachalinensis* have been traditionally used as conventional medicines (Sohn *et al.*, 2003; Kim and Lee, 2013). Especially, *R. japonica* was used for treatment of joint pains, menstrual cramps, maternity, cystitis, cancer (Kim *et al.*, 2008; Chung *et al.*, 2011), whereas *R. sachalinensis* was used in a laxative and a diuretic and analgesic treatment (Lajter *et al.*, 2013). However, in the markets, it is very difficult to distinguish these two species correctly because they are usually customized and purchased as the fragmented rhizomes types. Therefore, it is necessary to support the reproducible and easy protocols to evaluate the quality of crude medicinal resources.

In this study, we applied the ARMS method to establish the molecular identification methodology of *R. japonica* and *R. sachalinensis*. For this purpose, we analyzed and compared the sequence variation of seven chloroplast loci (*matK, atpB, atpF-H, trnF-trnL, accD-psal, psbK-I and rpl32-trnL*) among the two species. Based on the results, we developed the new molecular markers inferred from cpDNA for recognizing both species based on SNP sites.

Materials and Methods

Plant materials

The collection information for the used samples was summarized in Table 1. *R. japonica* was collected from four different areas of South Korea. Also, seven individuals of *R. sachalinensis* were sampled at Ulleung-do island. Voucher specimens were deposited at GCU (Herbarium, Gachon University). And we also extracted DNAs from dried specimens of KH (Herbarium of Korea National Arboretum), MPRB (Medicinal Plant Resources Bank for Immune and Metabolic Disease) and PDBK (Plant DNA Bank in Korea).

Table 1. Plant materials of this study

Scientific name	Serial No.	Voucher	Collecting sites
R. japonica	JA1	MPRB KR-07-00472	Cheongseong-myeon, Okcheon-gun, Chungcheongbuk-do
	JA2	LIH 2384	Gwangdo-myeon, Tongyeong-si, Gyeongsangnam-do
	JA3	HALLA 0775	Haean-dong, Jeju-si, Jeju-do
	JA4	S. C. Kim 20130915	Cheongseong-myeon, Okcheon-gun, Chungcheongbuk-do
	JA5	MPRB CN-09-00869	China
	JA6	PDBK 2005-1088	Seongpanak~the top of Hanla-san, Jocheon-eup, Bukjeju-gun, Jeju-do
	JA7	PDBK 2007-0504	Eorimok ~ Witse-Oreum ~ Yeongsil, Halla-san, Jeju-si, Jeju-do
	JA8	PDBK 2008-0548	Halla-San, Yeongsil, Jeju-si, Jeju-do
R. sachalinensis	SA1	Hana 141025	Bongnaepokpo, Jeodong-ri, Ulleung-eup, Ulleung-gun, Gyeongsangbuk-do
	SA2	KIOM 2013001	Ulleung-eup, Ulleung-gun, Gyeongsangbuk-do
	SA3	Ulleung 52-090624-019	Dodong-ri, Ulleung-eup, Ulleung-gun, Gyeongsangbuk-do
	SA4	Yoo 0265	Seonginbong , Dodong-ri, Ulleung-eup, Ulleung-gun, Gyeongsangbuk-do
	SA5	Hana 140820-1	Jeodong-ri, Ulleung-eup, Ulleung-gun, Gyeongsangbuk-do
	SA6	PDBK 2005-0671	Taeha-ryeong ~ Cheonbu-ri ~ Naeseugeon, Ullung-eup, Ullung-gun, Gyeongsangbuk-do
	SA7	PDBK 2012-1121	Guam~Taeharyeong~Taeha, Namseo-ri, Seo-myeon, Ulleung-gun, Gyeongsangbuk-do

DNA extraction and amplification of each loci

Total genomic DNA was extracted by following modified 2X CTAB buffer method (Doyle and Doyle, 1987). Lipids and other metabolites were removed using SEVAG solution [chloform (24) : isoamylalcohol (1)], and the DNA was precipitated with isopropanol at -20℃. The extracted DNA was then melted in 1X TE buffer and kept at -20℃. The DNA products were checked by 1% agarose gel electrophoresis with ethidium bromide staining and measured the concentration by spectrophotometer (biospec-nano; Shimadzu).

The information of primers used for amplification of each loci was shown in Table 2. PCR performed in 25 μl volume comprising 80 ng of template DNA, 0.1 U of e-*Taq* DNA polymerase (Solgent, Korea), 2 μl of 10X reaction buffer (100 mM Tris-HCl, 500 mM KCl, and 15 mM $MgCl_2$), 0.25 mM dNTPs, 2.5 mM $MgCl_2$, and 0.5 μM forward and reverse primers using a Perkin-Elmer 9700 machine (Perkin Elmer Applied Biosystems, Waltham, MA, USA). The thermo cycling profile consisted of 4 mins at 94℃, followed by 30-35 cycles of 2-3 mins at 94℃, 1 min 52-55℃, 1 min at 72℃, and an additional 7 mins extension at 72℃. The products were checked by 1% agarose gel electrophoresis with ethidium bromide staining and visualization under UV light.

Sequencing and alignment

All PCR products were purified using MeGa quick-spin total fragment DNA purification kit (Intron Biotechnology, Inc), and sequenced using cycle-sequencing BigDye Terminator Kit (V3.1, Life Technologies) based on the manufacturer's protocol. The high quality sequences were assembled and aligned using geneious v.7.1.8 (Biomatters Ltd., New Zealand).

The amplified sequences were aligned for finding the SNP sites in seven regions (Appendix 1). These regions were selected based on NCBI and CBOL Plant Working Group (2009).

Design of specific primers and PCR protocol

In *psbK-I* region, two internal primers (*psbK, psbI*)

Table 2. Primer sequences used for PCR in this study

loci	Primer	Sequence (5'-3')	Reference
atpB	atpB-1	5'-ACA TCK ART ACK GGA CCA ATA A-3'	Chiang *et al*., 1998
	rbcL-1	5'-AAC ACC AGC TTT RAA TCC AA-3'	Chiang *et al*., 1998
matK	matK-AF	5'-CTA TAT CCA CTT ATC TTT CAG GAG-3'	Yan *et al*., 2008
	matK-8R	5'-AAA GTT CTA GCA CAA GAA AGT CGA-3'	Yan *et al*., 2008
accD-psaI	accD	5'-AAT YGT ACC ACG TAA TCY TTT AAA-3'	Shaw *et al*., 2007
	psaI-75R	5'-AGA AGC CAT TGC AAT TGC CGG AAA-3'	Shaw *et al*., 2007
atp F-H	atpF	5'-ACT CGC ACA CAC TCC CTT TCC-3'	Lahaye *et al*., 2008
	atpH	5'-GCT TTT ATG GAA GCT TTA ACA AT-3'	Lahaye *et al*., 2008
trnL-F	trnL-5e	5'-GGT TCA AGT CCC TCT ATC CC-3'	Taberlet *et al*., 1991
	trnF-6f	5'-ATT TGA ACT GGT GAC ACG AG-3'	Taberlet *et al*., 1991
psb K-I	psbK	5'-TTA GCC TTT GTT TGG CAA G-3'	Lahaye *et al*., 2008
	psbI	5'-AGA GTT TGA GAG TAA GCA T-3'	Lahaye *et al*., 2008
rpl32-trnL	rpl32-F	5'-CGA TTC CAA AAA AAC GTA CTT C-3'	Shaw *et al*., 2007
	trnL-UAG	5'-CTG CTT CCT AAG AGC AGC GT-3'	Shaw *et al*., 2007

were designed to be used as a positive control, and a specific primer (psbKI-SNP-C) for the identification of *R. japonica* and its allies were designed on the basis of SNP. A similar strategy was applied for the *atpF-H* region in which a specific primer (atpFH-SNP-C) was designed. The reactions were run in a 25 μl volume containing 60-80 ng of template DNA, 0.1 U of e-*Taq* DNA polymerase (Solgent, Korea), 2.5 μl of 10X reaction buffer and 0.5 μl of 10 mM dNTPs. The amplification process consisted of 1 min at 94℃, followed by 25 cycles of 30 sec at 94℃, 15 sec at 60℃, and 30 sec at 72℃, with an additional 1 min extension at 72℃ for *psbK-I* and *atpF-H*.

Results

Sequences alignment

After seven loci or regions were sequenced, we measured the AT contents and counted the variable sites within each species and between species. Additionally, the percentages of variable sites on the loci were calculated (Table 3).

We found ten variable sites between the species and thirty-nine variable sites within populations of *R. japonica* and *R. sachalinensis,* respectively. But most of sites between the species were unuseful because those were located in high AT rich regions and terminals of regions. Therefore, we could selected two SNP sites from *psbK-I* and *atpF-H* regions which are effective to create molecular marker for identification of *R. japonica* and *R. sachalinensis,* respectively.

SNP primer specific to *R. japonica* of *psbK-I*

A PCR product of about 520 bp from *psbK-I* were amplified and sequenced. Within the populations of *R. japonica*, there was no sequence variation among individuals. Nevertheless, compared to individuals of two species including *R. sachalinensis*, we found two variable sites. And one of them could be used as species-specific SNP for *R. japonica* (Fig. 1).

To establish a molecular identification method for *R. japonica* and its related species, we designed a species-specific primer pair based on a SNP in the sequence that the second nucleotide from the 3'-end of the original sequence was modified. Specifically, the reverse primer psbKI-SNP-C (5'-CTC ACA AGG TCT TTC ACG

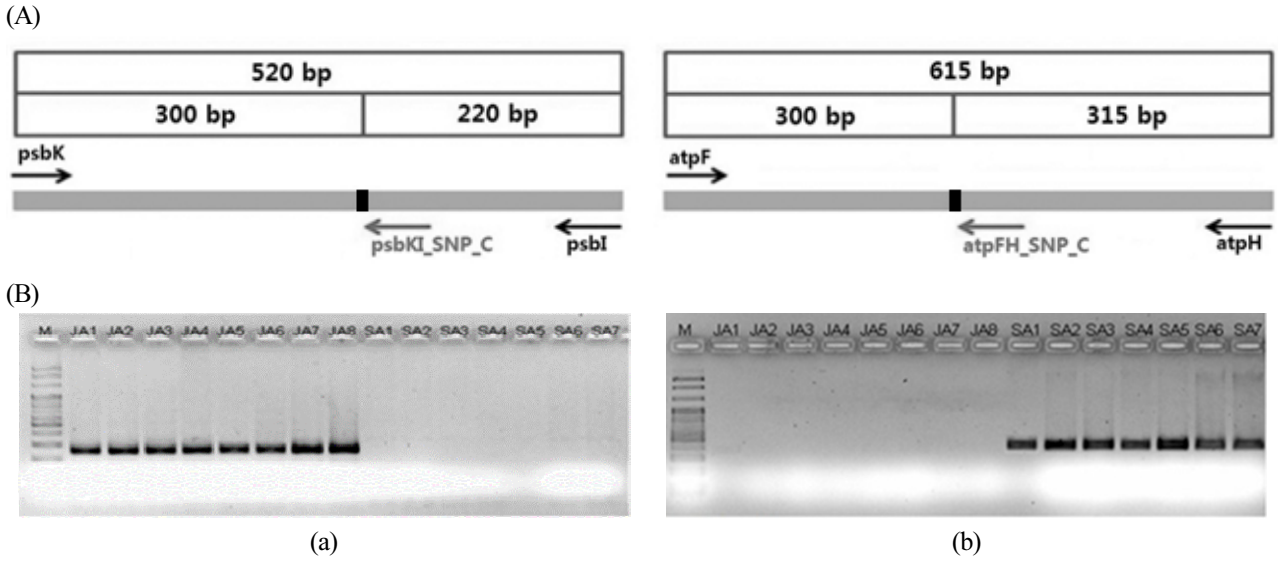

Fig. 1. (A) Schematic diagram of *psbK-I* and *atpF-H* region including the positions and size of the primers used for check reproducibility and availability of the species identification (psbK & psbKI_SNP_C; specific for *R. japonica* and atpF & atpF_SNP_C; specific for *R. sachalinensis*). (B) PCR products using newly designed primers (a) psbK & psbKI_SNP_C, (b) atpF & atpF_SNP_C. M: 100 bp DNA ladder; JA1-JA5: *R. japonica*; SA1-SA5 ; *R. sachalinensis*.

GCG-3') was designed to amplify the PCR products of *R. japonica* by replacing A with C. This modified primer did not work in *R. sachalinensis* (Fig. 1, Table 4).

SNP primer specific to *R. sachalinensis* of *atpF-H*

The complete *atpF-H* sequences from two species, 615 bps, were aligned. There were twenty two variation sites within *R. sachalinensis*, as well as thirty one variable sites among two species including twenty seven sites of insertion/deletion. From these results, we could find four species- specific SNPs sites for *R. sachalinensis* (Fig. 1).

Based on the *atpF-H* sequence variations, we designed a species-specific primer pair modifying an SNP located 300 bp which is a specific to *R. sachalinensis*. And the reverse primer atpFH-SNP-C (5'-TCG CAA TTT ACA CGA AAA CCC GCC-3') was designed to amplify *R. sachalinensis* by replacing G with C at the second nucleotide position from the 3' end of the primer. Also, this primer did not work for *R. japonica* (Fig. 1, Table 4).

Discussion

SNP is a specific site that has a higher variation rate resulting from small deletions or insertions in DNA sequences (Kim and Misra, 2007). Therefore, molecular marker using SNP sites contributes the development of an effective method for identification of close relative taxa. For example, Kim *et al.* (2013) reported the utilization of multiplex PCR to distinguish *Cynanchum wilfordii* (Maxim.) Hemsl. (白首烏; Eun-jo-rong, in Korean and ge shan xiao in Chinese), *C. auriculatum* Royle ex Wight (異葉牛皮消; niu pi xiao in Chinese) and *R. multiflora* (Thunb.) Moldenke (何首烏; Ha-su-o in Korean and he shou wu in Chinese). Similarly, the specific molecular marker for *S. chinensis*, *P. ginseng*, Tomato, *Zea mays* were developed (Batley *et al.*, 2002; Deynze *et al.*, 2007; In *et al.*, 2010; Kim *et al.*, 2012).

In this study, we have compared the seven loci (*atpB, matK, accD-psal, atpF-H, trnL-trnF, psbK-I* and *rpl32-trnL*) from chloroplast DNA sequences to establish the molecular markers for distinguishing *R. japonica, R.*

Table 3. The information of investigated sequences

Region	AT Cont. (%)	Total length (bp)	No. of variable sites within species		No. of variable sites between species
			R. japonica	*R. sachalinensis*	
*atp*B	69.5	761	5 (0.66%)	0 (0.00%)	0 (0.00%)
*mat*K	66.2	1,199	12 (1.00%)	9 (0.75%)	0 (0.00%)
*acc*D-*psa*I	73.5	844	4 (0.47%)	0 (0.00%)	0 (0.00%)
*atp*F-H	64.1	615	1 (0.15%)	0 (0.00%)	4 (0.60%)
*trn*L-F	63.7	449	1 (0.22%)	2 (0.45%)	0 (0.00%)
*psb*K-I	67.3	520	0 (0.00%)	1 (0.19%)	2 (0.37%)
*rpl*32-*trn*L	72.9	763	6 (0.79%)	2 (0.26%)	4 (0.52%)

Table 4. The sequence of species-specific primer pair based on an SNPs

Loci	Primer	Sequence (5'-3')	Tm (℃)	specificity
psbK-I	psbKI_SNP_C	5‘-CTCACAAGGTCTTTCACGGCG-3’ (A→C)	63.3℃	*R. japonica*
atpF-H	atpFH_SNP_C	5‘-TCGCAATTTACACGAAAACCCGCC-3’ (G→C)	65.3℃	*R. sachalinensis*

sachalinensis and related taxa. Generally, the sequence variations are more frequent in non-coding and intron regions than protein- coding sequences (Ching *et al.*, 2002; Van Deynze *et al.*, 2007). However, intron regions are conserved among species, therefore, it is more effective to design primers within non-coding regions than intron regions for SNP study (Fourmann *et al.*, 2002). Based on previous studies, five intergenic spacer regions of *accD-psaI*, *atpF-H*, *trnL-F*, *psbK-I*, *rpl32-trnL* and two highly variable coding regions of *atpB* and *matK* were selected in this study (Taberlet *et al.*, 1991; Chiang *et al.* 1998; Shaw *et al.*, 2007; Lahaye *et al.*, 2008; Yan *et al.*, 2008). The results showed different numbers of variable sites within *R. japonica* and *R. sachalinensis* (Table 3). Especially, coding region of *matK* showed the highest number of different sites (12 sites in *R. japonica* and 9 sites in *R. sachalinensis*) followed by the *rpl32-trnL* region (6 sites in *R. japonica* and 2 sites in *R. sachalinensis*).

Among the investigated sequence regions, we found two species-specific SNPs in *psbK-I* and *atpF-H* regions. Although the variations were observed within species of *R. japonica* and *R. sachalinensis*, it was not so different between these two species except the *atpF-H*, *psbK-I* and *rpl32-trnL* (Table 3). This result suggested the close relationship of *R. japonica* and *R. sachalinensis*. Additionally, it was successful to design SNP primers specific to *R. japonica* with *psbK-I* and to *R. sachalinensis* with *atpF-H* (Fig. 1). These specific primers, which were designed based on ARMS, amplified 300 bp specific fragment in each region. Previously, the circumscription of *R. japonica* and *R. sachalinensis* remained controversy because of their complex morphological characters (Steward 1930; Bailey and Stace, 1992; Kim and Park 2000; Park *et al.*, 2011). Furthermore, *R. japonica* and *R. sachalinensis* have been used confusedly for the treatment of menstrual cramps, cancer, chronic bronchitis and so on (Kim *et al.*, 2008; Chung *et al.*, 2011). Therefore, it is necessary to establish the distinguishing protocols using molecular biology technique for methodical identification of useful medicinal herbs.

In this study, it was able to identify *R. japonica* and *R. sachalinensis* based on molecular marker from *psbK-I* and *atpF-H* regions, respectively. And these newly designed molecular markers could be applied effectively to recognize both species. These molecular markers will be useful information for detection of genotyping maker for protection as well as digitalization for

identification of cultivars. Furthermore, the protocol described in this study should be useful for further studies focusing an identification of economically important species. Generally, it can be applied to promote the value of plant resources and to increase the needs on modernized discrimination protocol of natural products.

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Appendix 1. List of sequences used for alignment to find the SNPs site in seven regions

Species	Voucher	*atpB*	*matK*	*accD-psaI*	*atpF-atpH*	*trnL-trnF*	*psbK-psbI*	*rpl32-trnL*
R. japonica	MPRB KR-07-00472		O		O	O	O	O
	LIH 2384		O	O	O	O	O	O
	HALLA 0775		O	O	O	O	O	O
	S. C. Kim 20130915		O	O	O		O	O
	MPRB CN-09-00869						O	O
		EF017665	AYO42586	HM137519		EU024786		JN235020
		HQ843097	EF153700			JN235062		
		JN234939	GU373522					
			HM357919					
			EU024772					
R. sachalinensis	Hana 141025					O		
	KIOM2013001	O	O	O	O		O	O
	Ulleung 52-090624-019	O	O	O	O	O	O	O
	Yoo 0265	O	O	O	O		O	O
	Hana 140820-1		O		O	O	O	O
		EF017666	EF438009			JF831320		
			AY042635					
Total sequence No.		7	15	7	8	9	9	10