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ORIGINAL ARTICLE

Genus identification of toxic plant by real-time PCR

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Abstract Some plants have toxicities that are dangerous for humans. In the case of poisoning by toxic plants, a rapid and easy screening test is required for accurate medical treatment or forensic investigation. In this study, we designed specific primer pairs for identification of toxic plants, such as subgenus *Aconitum*, genus *Ricinus*, genus *Illicium*, and genus *Scopolia*, by internal transcribed spacer sequences of nuclear ribosomal DNA. Allied species of target plants, foods, and human DNA were not detected, but each primer pair provided a specific PCR product from the target plant using real-time PCR. This method can detect the subgenus *Aconitum*, genus *Ricinus*, and genus *Scopolia* with template DNA of 10 pg, respectively, and genus *Illicium* with 1 pg. Furthermore, each primer pair provided the exact PCR product from digested target plants in artificial gastric fluid. When a trace unknown plant sample in forensic investigation is collected from stomach contents, this PCR assay may be useful for screening toxic plants.

Keywords *Aconitum* · *Illicium* · *Ricinus* · *Scopolia* · Internal transcribed spacer · Real-time PCR

Introduction

Some plants have toxicities that are dangerous for humans. Poisoning is most frequently caused by accidental ingestion, as it can be mistaken for an edible plant. *Aconitum* sp., *Illicium anisatum* and *Scopolia* sp., which are highly toxic, are similar to edible plants that grow wild in Japan. A few people die from accidental ingestion of *Aconitum* sp., which resemble *Anemone flaccid* Fr. Schm., *Cirsium dipsacolepis*, and *Epetrium nigrum* in morphology [1–3]. Seeds of *I. anisatum*, which are designated as deleterious substances in the Poisonous and Deleterious Substances Control Law in Japan, are eaten because they appear similar to seeds of sii [4]. *Scopolia* sp. are also mistaken for food plants found on mountain sides [5].

As they are highly toxic, *Aconitum* sp. and *Ricinus communis* are sometimes used in suicides and homicides [6–9]. *R. communis* is the source of castor oil, which has broad applications in various industries. Ricin, produced from the seeds of *R. communis*, is the only protein listed in the Chemical Weapons Convention [10]. Its toxicity has a typical mouse LD_{50} in the order of 2 μg/kg body weight, intraperitoneally [11]. In addition, undetected homicides are disclosed by forensic exhumations [12]; however, at autopsy, toxic plants are not included in the routine screening for toxicological analysis. Easy screening of toxic plants can contribute to identify undetected homicides. In all cases, such as accidental ingestion, suicides, homicides, and undetected homicides, a rapid and easy screening test of toxic plants is required for accurate medical treatment or forensic investigation.

Generally, methods to identify toxic plants have involved morphological analysis, gas chromatography, and HPLC/MS [13–18]; however, these methods are time consuming and are complicated for identification. A trace toxic sample is difficult to detect by morphological analysis.

Among the molecular technologies, the identification system of general plants to species level is known as DNA barcoding, which consists of a standardized short sequence of DNA with the universal primer [19–21]. The DNA

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barcoding method was developed in the forensic field [22, 23]; however, it is difficult to isolate the target sample from the mixtures with the universal primer. In other molecular technology, a quantitative real-time PCR assay for hallucinogenic plants in plant mixtures has been reported [24]. This quantification assay is based on the relative quantification of DNA extracted from a mixture versus reference DNA extracted from a known amount of pure target plant.

In this study, we tried to develop a novel, specific, and easy identification system of toxic plants using real-time PCR. An advantage of real-time PCR over gas chromatography and HPLC/MS is detecting toxic plants without a standard sample. Moreover, real-time PCR is known as a highly sensitivity assay [25]; thus, it may detect and quantify trace and mixed toxic samples that cannot be identified using traditional methods.

Methods

Materials

Subgenus *Aconitum* (*Aconitum hakusanense*, *Aconitum japonicum*, *Aconitum sanyoense*, and *Aconitum senanense*), genus *Illicium* (*I. anisatum* and *Illicium verum*), genus *Ricinus* (*R. communis*), and genus *Scopolia* (*Scopolia japonica*) were chosen as the target toxic plants in this study. Target plants and allied plants are shown in Table 1. Specimen materials have been stored in the Garden of Medicinal Plants, Kyoto Pharmaceutical University. The wild types of Code wR002, wC001, wC002, and wAh001 were provided by Uji City Botanical Park. Drug materials were obtained from Tochimototenkaido (Osaka, Japan). Market material was purchased from a flower shop. General foods, such as rice, Irish potato, tomato, eggplant, cabbage, carrot, lettuce, onion, nap, cucumber, Satsuma orange, broccoli, radish, green pepper, white turnip and apple, and 9947A DNA (Promega, Madison, WI, USA) as human DNA were tested to evaluate the primer specificity.

DNA extraction

Plant materials were frozen at −80°C and ground to a fine powder with Multi-beads Shocker (Yasuikikai, Tokyo, Japan). DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Extracted DNA was quantified by optical absorbance at 260 nm.

Sequencing analysis and primer design

The internal transcribed spacer (ITS) 1, 5.8S ribosomal RNA (rRNA) gene, and ITS2 region of plant materials (sA521, sA522, wA001, wA005, wA006, wI001, wI002, wScC001, sSc710, wR001 wM001, wS001, and wS002) were sequenced using universal primers with an ABI Genetic Analyzer and a BigDye™ Terminator Cycle Sequencing Kit ver.1.1 and 3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions [26]. On the basis of the ITS sequences from target plants and data from the National Center for Biotechnology Information (NCBI), the four primer pairs were designed for the specific amplification of subgenus *Aconitum*, genus *Illicium*, genus *Ricinus*, and genus *Scopolia* (Table 2).

PCR conditions

Polymerase chain reaction (PCR) was carried out in a 12-μl reaction mixture containing 6 μl One Shot LA PCR™ Mix (Takara, Shiga, Japan), 0.5 μM primer pairs, and template DNA. PCR amplification was performed in a GeneAmp PCR system 9700 (Applied Biosystems) programmed for 1 min at 94°C; 25 cycles of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, and a final extension for 5 min at 72°C. PCR products were confirmed by electrophoresis for 40 min at 100 V in a 2.0% agarose gel and directly visualized with ethidium bromide under UV light.

Real-time PCR conditions

Real-time PCR was carried out in a 25-μl reaction mixture containing 12.5 μl SYBR Premix Ex Taq™ (Takara), 0.5 μM primer pairs, and template DNA. Amplification was performed in a SmartCyclerII (Cepheid, Sunnyvale, CA, USA) programmed for 10 s at 95°C; 35 cycles of 5 s at 95°C, 20 s at 58°C, and 20 s at 72°C. After amplification, melt curve analysis was performed by heating the reaction mixture from 65°C to 95°C at a rate of 0.2°C/s.

Forensic specimens

Target plants were digested in artificial gastric fluid as false forensic samples. Artificial gastric fluid was made from 2.0 g sodium chloride and 3.2 g pepsin in 7.0 ml hydrochloric acid and water up to 1,000 ml [27]. The pH of this test solution was about 1.2. Each crushed target plant weighed 100 mg and was incubated in artificial gastric fluid at 37°C for 4 h. Extracted DNA from digested and untreated samples was confirmed by electrophoresis for 40 min at 100 V in a 2.0% agarose gel and directly visualized with ethidium bromide under UV light. Extracted DNA from digested toxic plants was tested by real-time PCR using each primer pair of aco15F/aco18R, ill30F/ill34R, ric49F/ric53R, and sco07F/sco08R.

Table 1 Plant materials

Family	Genus	Subgenus	Species	Type	Code	Place of collection	Notes
RANUNCULACEAE	*Aconitum*	*Aconitum*	*A. hakusanense*	specimen	sA520	Nagano, Japan	Target plant
			A. japonicum	specimen	sA521	Kyoto, Japan	Target plant
			A. japonicum	specimen	sA522	Shiga, Japan	Target plant
			A. japonicum	specimen	sA523	Shiga, Japan	Target plant
			A. sanyoense	specimen	sA527	Hyogo, Japan	Target plant
			A. senanense	specimen	sA528	Osaka, Japan	Target plant
			A. senanense	specimen	sA529	Shiga, Japan	Target plant
			A. species	wild	wA001	Kyoto, Japan	Target plant
			A. species	wild	wA002	Kyoto, Japan	Target plant
			A. species	wild	wA005	Shiga, Japan	Target plant
			A. species	wild	wA006	Hokkaido, Japan	Target plant
		Lycoctonum	*A. pterocaule*	specimen	sL524	Shiga, Japan	Allied plant
			A. chrysopilum	specimen	sL525	Shiga, Japan	Allied plant
	Anemone		*A. nikoensis*	specimen	sAne422	Kyoto, Japan	Allied plant
			A. flaccida	specimen	sAne465	Kyoto, Japan	Allied plant
	Pulsatilla		*P. cernua*	wild	wP001	Kyoto, Japan	Allied plant
	Cimicifuga		*C. simplex*	specimen	sCi903	Ishikawa, Japan	Allied plant
	Helleborus		*H. species*	wild	wH001	Kyoto, Japan	Allied plant
ILLICIACEAE	*Illicium*		*I. anisatum*	specimen	sI461	Kyoto, Japan	Target plant
			I. anisatum	wild	wI001	Kyoto, Japan	Target plant
			I. anisatum	wild	wI002	Kyoto, Japan	Target plant
			I. anisatum	wild	wI003	Kyoto, Japan	Target plant
			I. anisatum	wild	wI004	Kyoto, Japan	Target plant
			I. anisatum	wild	wI005	Kyoto, Japan	Target plant
			I. anisatum	wild	wI006	Kyoto, Japan	Target plant
			I. anisatum	market	wI007	No data	Target plant
			I. verum	wild	wIv001	Kyoto, Japan	Target plant
SCHISANDRACEAE	*Schisandra*		*S. chinensis*	wild	wScC001	Kyoto, Japan	Allied plant
			S. nigra	specimen	sSc710	Kyoto, Japan	Allied plant
	Kadsura		*K. japonica*	wild	wK001	Kyoto, Japan	Allied plant
EUPHORBIACEAE	*Ricinus*		*R. communis*	wild	wR001	No data	Target plant
			R. communis	wild	wR002	Kyoto, Japan	Target plant
			R. communis	drug	dR01	Vietnam	Target plant
	Mallotus		*M. japonicus*	wild	wM001	Kyoto, Japan	Allied plant
			M. japonicus	drug	dM01	Tokushima, Japan	Allied plant
	Codiaeum		*C. variegatum*				
			Müll.Arg.var.*pictum* cv.	wild	wC001	Kyoto, Japan	Allied plant
			C. variegatum	wild	wC002	Kyoto, Japan	Allied plant
			Müll.Arg.var.*pictum* cv.				
	Acalypha		*A. hispida*	wild	wAh01	Kyoto, Japan	Allied plant
SOLANACEAE	*Scopolia*		*S. japonica*	wild	wS001	Kochi, Japan	Target plant
			S. japonica	wild	wS002	Kochi, Japan	Target plant
			S. japonica	wild	wS003	Japan	Target plant
			S. japonica	wild	wS004	Japan	Target plant
			S. japonica	specimen	sS302	Gifu, Japan	Target plant
			S. japonica	specimen	sS303	Shiga, Japan	Target plant
			S. japonica	specimen	sS304	Kyoto, Japan	Target plant
	Atropa		*A. belladonna*	drug	dA01	No data	Allied plant

Table 2 Primer sequences for target plants

Targeted genus	Name	Sequence(5′–3′)	Product length (bp)
Aconitum	aco15F aco18R	CCCGTCAACCACGTTGTCGG GTCGATGTGTCCCAACGTGCAA	171
Illicium	ill30F ill34R	ACTAGGCTAACCGGCGAACT ATTCGGGTCTACAGCACCAC	234
Ricinus	ric49F ric53R	AACATGTTTGCTTATTGCAAGG AGCAGCGTGCTCTTTTCATTTAAT	168
Scopolia	sco07F sco08R	GCGGGCGGCTGACTAACGAA CGTCGAGGCGCGCTGTCAAT	72
universal for ITS region	18s-26s-F1 18s-26s-R1	GTAGGTGAACCTGCGGAAG GGTAGTCCCGCCTGACCT	Variable
	ITS-A[a] ITS-C[a]	GGAAGGAGAAGTCGTAACAAGG GCAATTCACACCAAGTATCGC	Variable
	ITS-B[a] ITS-D[a]	CTTTTCCTCCGCTTATTGATATG CTCTCGGCAACGGATATCTCG	Variable

[a] Blattner FR et al. [26]

Results

Primer specificity

Each primer pair of aco15F/aco18R, ill30F/ill34R, ric49F/ric53R, and sco07F/sco08R specifically amplified the 1 ng template DNA of the target plant, respectively (Fig. 1). No sizing discrepancy was found between the PCR product and the expected result from direct sequencing analysis. Negative controls, allied plants in Table 1, the examined foods, and human DNA were not detected with each primer pair (data not shown).

Sensitivity of detection real-time PCR

The sensitivity of real-time PCR was evaluated using DNA from the target plants serially diluted from 1 ng to 0.1 pg. The limits of detection of subgenus *Aconitum*, genus *Ricinus*, and genus *Scopolia* were 10 pg and that of genus *Illicium* was 1 pg (Fig. 2a–d). The specific melting points of *Aconitum*, *Illicium*, *Ricinus*, and *Scopolia* were approximately 91.2±0.2°C, 86.6±0.2°C, 91.8±0.2°C, and 87.3±0.2°C, respectively. In the subgenus *Aconitum* assay, the melting point of the negative control was approximately 80.69°C.

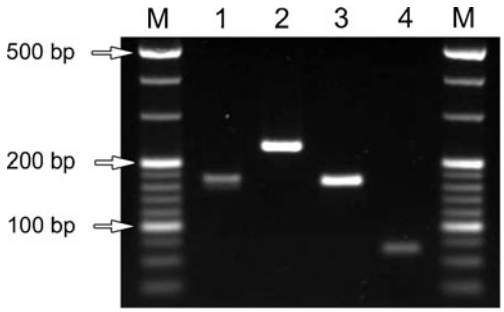

Fig. 1 Specificity of the primer pairs PCR products with primer pairs using 1 ng DNA from target plants were as follows: *lane M* 20 bp ladder marker (Takara); *lane 1* amplified with aco15F/aco18R using DNA of *Aconitum*; *lane 2* amplified with ill30F/ill34R using DNA of *Illicium*; *lane 3* amplified with ric49F/ric53R using DNA of *Ricinus*; *lane 4* amplified with sco07F/sco08R using DNA of *Scopolia*

Forensic specimens

The extracted DNA recoveries of digested target plants were less than those of untreated samples (Fig. 3a). Although the extracted DNA from digested samples was too small to quantify accurately, each primer pair of aco15F/aco18R, ill30F/ill34R, ric49F/ric53R, and sco07F/sco08R detected toxic plants from the 2 μl extracted solution of digested samples by real-time PCR (Fig. 3b). The melting point of *Aconitum* negative control was approximately 80.59°C, and those of other negative controls were not detected.

Discussion

In general, forensic botany is not very common; however, it can help in some forensic cases. Botanical evidence in establishing the minimum postmortem interval of human remains using the growth rate of bryophytes and plant roots [28], and the identification of the toxic plant in causing poisoning plays an important role in forensic investigations. The DNA barcoding method can be widely applied to forensic botany without traditional botanical morphology and correctly identify to species level by a multilocus approach; however, the universal primer's intrinsic property of allowing amplification across a wide taxonomic range of species complicates the identification of mixtures [22, 23]. In forensic investigations, it is difficult to detect the target plant using a universal primer from the stomach contents, which consist of various plants as foods. In other words, the major components of plant foods only are amplified by the universal primer and minor components of target plant cannot be amplified enough. Furthermore, Lee et al. in a

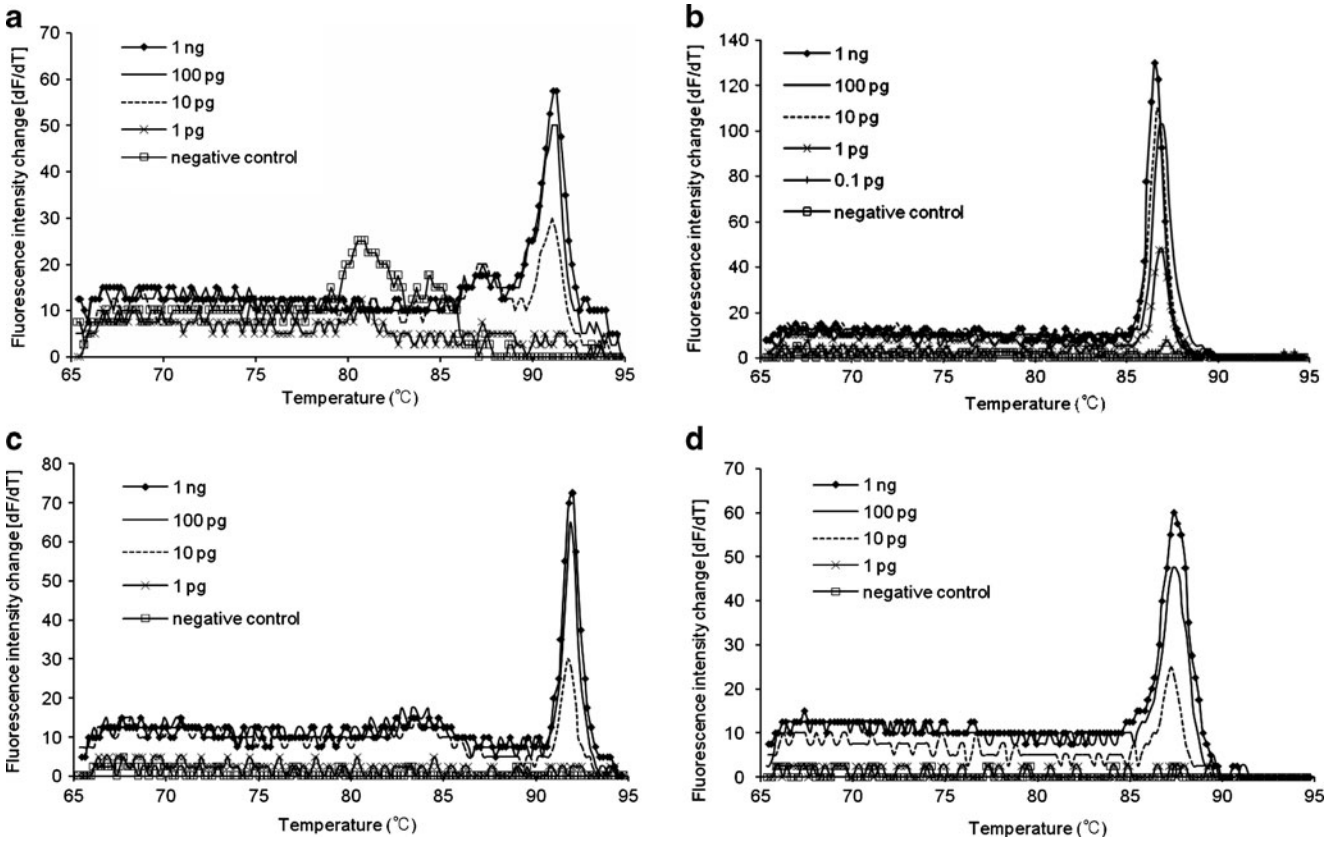

Fig. 2 Sensitivity of the primer pairs by real-time PCR. The amount of template DNA from target plants changed the fluorescence intensity and each target plant had its original melting point during real-time PCR with specific primer pairs. **a** *Aconitum*; **b** *Illicium*; **c** *Ricinus*; **d** *Scopolia*

recently published case report, in which they performed DNA and chemical analysis of the gastric content of a deceased person, concluded that universal markers, including ITS and 18S rRNA, were unsuitable for the DNA analysis of gastric contents because a universal primer could bind to the ITS and 18S rRNA of humans [29]. A few of the gastric epithelial cells from the victim remained mixed with the plant cells, which made it difficult to determine the ITS and 18S rRNA sequences of the plant evidence via direct sequencing. Thus, a specific primer is essential for amplification of the toxic plant.

The aim of this study was to amplify toxic plants specifically using the ITS region of nuclear ribosomal DNA. The ITS region is one of the most common regions for phylogenetic inference at the generic and infrageneric levels in plants [30]. The genus *Aconitum*, which consists of about 300 species, is well known for its extreme difficulty in classification by morphological analysis [31]. Identification of toxic plants at the subgenus level using real-time PCR is thought to be effective. The primer target site in this study was about the same as that in Luo et al.'s sequences of 51 *Aconitum* sp. [32]. The aco15F/aco18R primer pair may detect other species of subgenus *Aconitum*. On the other hand, the genus *Ricinus*, which has only one species, *R. communis*, does not need species level analysis. In the case of genus *Illicium*, *I. anisatum* has high toxicity, but other nontoxic species, such as *I. verum*, are used as spices in many countries. In this study, we could detect the genus *Illicium* but could not distinguish *I. anisatum* from the genus *Illicium*. The restriction fragment length polymorphism (RFLP) method to distinguish between *I. anisatum* and other *Illicium* sp. was reported by Techen et al. [33]. If species level analysis from genus *Illicium* is needed, we think that the combination of real-time PCR with the RFLP method overcomes the problem of identifying *I. anisatum* among forensic specimens. Genus *Scopolia* has about 12 species according to the database of The International Plant Names Index. In this study, the sample of genus *Scopolia* was only *S. japonica*. There is a large difference in the ITS region among *S. japonica*, *S. carniolica*, *S. parviflora*, and *S. lutescens* in the NCBI database. Moreover, the molecular phylogeny of Solanaceae in chloroplast DNA supports that *S. japonica* is different from *S. carniolica* [34]; therefore, the primer pair of sco07F/sco08R can potentially specifically, detect *S. japonica*.

No detection of negative controls, allied plants, foods, and human DNA in the electrophoresis of PCR products

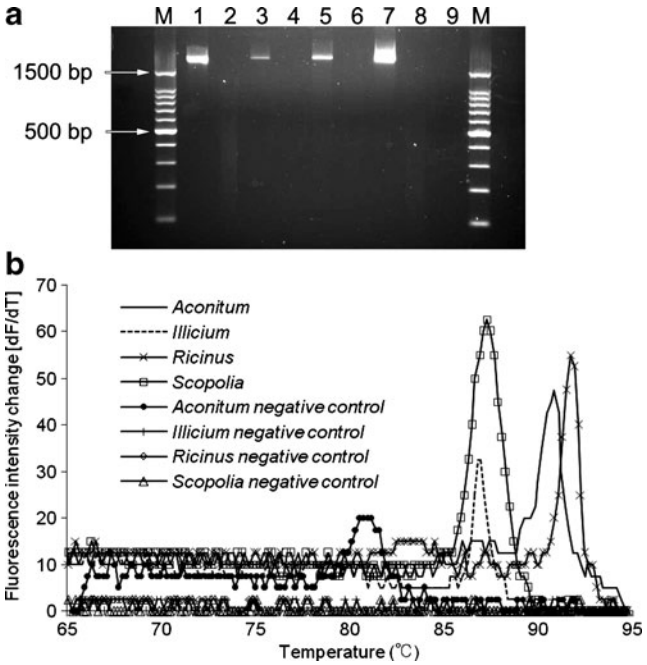

Fig. 3 **a** DNA size of digested target plants in artificial gastric fluid Extracted DNA was as follows: *lane M* 100 bp ladder marker (Takara); *lane 1* no treatment of *Aconitum*; *lane 2* digestion from *Aconitum*; *lane 3* no treatment of *Illicium*; *lane 4* digestion from *Illicium*; *lane 5* no treatment of *Ricinus*; *lane 6* digestion from *Ricinus*; *lane 7* no treatment of *Scopolia*; *lane 8* digestion from *Scopolia*; *lane 9* negative control. **b** Detection of digested target plants using real-time PCR Extracted DNA shown in (**a)** lanes *2*, *4*, *6*, and *8* were amplified with each primer pair

suggested that each primer pair was specific; however, the negative control of real-time PCR with aco15F/aco18R primer pair was amplified and the approximately 80.6± 0.2°C melting point was thought to indicate a primer dimer. The melting points of *Aconitum*, *Illicium*, *Ricinus*, and *Scopolia* were above 85°C and were different from that of the negative control in the *Aconitum* assay. Each melting point was a primer-specific temperature, and if this assay amplifies unrelated target sites of unknown samples, target plants will be distinguished from others by primer-specific temperatures.

The advantage of using the ITS region for the assay is its frequent repetition in the plant nuclear genome along with the other components of nuclear DNA [35]. This high copy number provides higher sensitivity than one copy number. In fact, this real-time PCR assay detection limit was 10 or 1 pg. These data indicated that this assay was highly sensitive and useful for forensic trace samples. In particular, the examination of stomach contents to find the cause of death is a very important aspect of forensic investigation; however, it is difficult to identify unknown plants by morphological analysis because digested plants are trace and mixed. We could detect a target plant from a trace sample in artificial gastric fluid (Fig. 3b). When a trace unknown plant sample is mixed with other foods and human cells in stomach contents, this specific primer using real-time PCR may be useful for screening toxic plants. The next step is the detection of a target plant from mixed samples in forensic investigation and confirming the results of this study under experimental conditions in real casework.

In Japan, subgenus *Aconitum*, genus *Illicium*, genus *Ricinus*, and genus *Scopolia* are natural growth plants that are easy to obtain. These plants can cause fatal poisoning due to accidental ingestion. In a future study, our method needs further improvements and developments with the design of new primer pairs to cover the widest geographical distribution, as now it can be applied only to the screening of the genus tested. In addition, we will improve this method to shorten the analysis time by direct PCR without the DNA extraction phase and to identify accurately by designing new specific primer pairs which target internal standard. According to the method of Luciano et al. [24], external genomic DNA from a general plant helps to quantify the target plant accurately in plant mixtures, as an internal standard, at the beginning of DNA extraction. External genomic DNA shows the existence of an inhibitor in plant mixture and eventual errors derived from laboratory manipulation. We will try to develop this real-time PCR assay to identify plants in cases of mixed and traced specimens, as in stomach contents, and for the quantification of a given plant species in biological mixtures.

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