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ORIGINAL PAPER

Real-Time PCR Quantification of Chloroplast DNA Supports DNA Barcoding of Plant Species

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Abstract Species identification from extracted DNA is sometimes needed for botanical samples. DNA quantification is required for an accurate and effective examination. If a quantitative assay provides unreliable estimates, a higher quantity of DNA than the estimated amount may be used in additional analyses to avoid failure to analyze samples from which extracting DNA is difficult. Compared with conventional methods, real-time quantitative PCR (qPCR) requires a low amount of DNA and enables quantification of dilute DNA solutions accurately. The aim of this study was to develop a qPCR assay for quantification of chloroplast DNA from taxonomically diverse plant species. An absolute quantification method was developed using primers targeting the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene using SYBR Green I-based qPCR. The calibration curve was generated using the PCR amplicon as the template. DNA extracts from representatives of 13 plant families common in Japan. This demonstrates that qPCR analysis is an effective method for quantification of DNA from plant samples. The results of qPCR assist in the decision-making will determine the success or failure of DNA analysis, indicating the possibility of optimization of the procedure for downstream reactions.

Keywords Quantitative real-time PCR · DNA barcoding · Ribulose bisphosphate carboxylase large subunit gene · Botanical samples

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Introduction

Plants grow in areas of human habitation worldwide and many have been used for human benefit. Taxonomic identification of plant samples to species level is often required. For example, some edible plants may cause a severe allergic reaction and detection of the allergen in a food item is important [1, 2]. Correct identification of plant samples at an archeological excavation contributes to a better understanding of ancient human life [3, 4]. In criminal investigations, if tiny plant fragments found on a suspect can be proven to originate from a crime scene, they can be an important evidence to associate the suspect with the site. In addition, taxonomic determination of an unidentified plant sample taken from a victim serves to identify the area where a crime was committed [5–8]. Such botanical samples are often damaged and fragmentary, preventing accurate species identification from morphological characteristics [6]. Applications of DNA-based techniques are powerful tools in a diversity of biological fields. DNA barcoding, which involves the use of a short region of DNA from botanical samples for species identification, is commonly used [5, 9–12]. An official recommendation for plant barcoding was established using regions of the chloroplast genome [11], and large genetic databases such as GenBank [13] and the Barcode of Life Data Systems (BOLD) [14] are readily accessible.

It is not usually necessary to quantify genomic DNA extracts for DNA barcoding because the samples used for phylogenetic and taxonomic studies are fresh and sufficient for PCR amplification [15, 16]. Ultraviolet–Visible spectrophotometry (UV–Vis) was adopted as the quantitation method for DNA barcoding in other studies [17, 18]. However, DNA quantification is required for an accurate and effective examination. If a quantitative assay provides

unreliable estimates, a quantity of DNA higher than the estimated amount may be used in a subsequent examination to avoid failure to analyze samples from which DNA extraction is difficult or insufficient sample DNA hinders the opportunity to perform additional analyses. Furthermore, some methods, such as short tandem repeat analysis, require strict control of input DNA [19, 20]. Conventional DNA quantification methods, for example UV–Vis and fluorometry, often fail to quantify dilute DNA solutions accurately because of the influence of nonspecific background noise. Such methods also cannot differentiate between DNA from botanical samples and that from human and environmental microbial contaminants. In addition, an important problem with botanical evidence is the low yield of DNA from damaged samples. Conventional methods are unable to quantify dilute DNA solutions and require a relatively large amount of DNA.

A method for quantification of DNA that is difficult to isolate is also of importance in forensic science. To address this issue, human DNA is quantified by real-time PCR [21–28]. Quantitative real-time PCR (qPCR) requires only a small amount of DNA and is able to quantify a dilute DNA solution accurately [21, 27, 28]. This method enables target-specific quantification based on primer design [22, 25, 26] and assists in the decision-making that will determine the success or failure of DNA analysis. The advantages of qPCR may make it suitable for quantification of botanical DNA from damaged samples. Previous research reported that qPCR is useful for plant species identification with quantification, even if the samples are damaged [29–33]. In addition, if qPCR assists in the decision-making that determines the success or failure of DNA analysis, the amount of DNA needed for additional analysis can be estimated [23].

Quantification of the amount of chloroplast DNA, which is the target of downstream reactions, is appropriate to determine whether the reactions can be optimized based on the estimated DNA quantity. Millions of chloroplasts are contained within a plant body and the number per cell varies, for example with tissue development and aging and among species [34–36]. A previous report described a method for relative quantification of chloroplast genome copies to study the expression of organellar genes and the genome copy number was expressed as copies relative to a nuclear reference gene [34–37]. However, this method requires several rounds of PCR amplification and results in loss of sample DNA. An absolute quantification method, which is used for quantification of human DNA in forensic science [24], is required for quantification of chloroplast DNA.

In this study, we developed an absolute quantification method for chloroplast DNA using primers targeting the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene with SYBR Green-based qPCR. The calibration curve was obtained using the PCR amplicon as the template. Taxonomically diverse plant DNA samples were analyzed successfully, demonstrating that qPCR analysis is an effective method with which to quantify DNA from botanical samples. The results of qPCR will assist in the decision-making that will determine the success or failure of DNA analysis, indicating the possibility of optimization of the procedure for downstream reactions.

Materials and Methods

Samples

Whole plants of *Arabidopsis thaliana* (ecotype Col-0) (Inplanta Innovations Inc., Yokohama, Japan) were used for development of the qPCR analysis method. Leaf samples of 15 common plant species widely distributed in Japan were collected at Miyazaki Prefecture in 2010. All samples were stored at room temperature.

Total genomic DNA was extracted from each ∼100-mg (wet weight) sample using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The DNA extracts were stored at -20 °C until analysis. The DNA concentration for each extract was measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Inc., Waltham, MA, USA).

Real-Time PCR Amplification of *rbcL* Fragment

Real-time PCR experiments for all samples were conducted with a SmartCycler II System (Takara, Otsu, Japan). A partial *rbcL* sequence was amplified by PCR using the primer set rbcL-F3 (5′-TATCTTGGCAGCATTCCGAGT AACTCC-3′) and rbcL-R3 (5′-GATTCGCAGATCCTC CAGACGTAGAGC-3′) [7, 38]. Primers were designed from the *rbcL* sequences for universal amplification of a 316-bp fragment from common plant species to estimate the optimal amount of DNA that can be used for DNA barcoding.

Each PCR was performed in a total volume of 25 μL that contained 200 μM dNTPs, 0.01 % bovine serum albumin (BSA), 0.2 μM each primer, 0.15 U Ex*Taq* DNA polymerase (Takara), 1× ExTaq reaction buffer, 0.1× SYBR Green I (Cambrex Corporation, East Rutherford, NJ, USA), and DNA template. The DNA template consisted of serial fivefold dilutions of *A. thaliana* genomic DNA or 1 μL of DNA extract from other plant species sampled.

The PCR amplicon, which was the same region used for qPCR, was used for generation of a standard curve because it was assumed that a standard curve cannot be obtained from samples when only a trace amount of DNA can be extracted from a sample. The PCR amplicon was amplified

using *A. thaliana* DNA as previously described [7] and purified with the QuickStep2 PCR Purification Kit (Edge BioSystems, Gaithersburg, MD, USA). The purification procedure used resin and gel filtration cartridges to remove primers, DNA, enzymes, salts, dNTPs, and other small molecules, which ensured PCR product purity prior to quantification using the UV–Vis method. A standard curve was obtained for the PCR amplicon from 10^3 to 10^7 chloroplast copies. The copy number was calculated as follows: mass in Daltons (g/molecule) = (size of dsDNA (bp) × 330 Da × 2)/Avogadro's number [39]. Water blanks were used as a negative control.

The PCR conditions were as follows: 98 °C for 1 min, then 35 cycles of 98 °C for 10 s, 56 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 5 min. The amplification specificity was checked by melting curve analysis of the PCR products performed by ramping the temperature to 95 °C for 15 s and back to 60 °C for 60 s followed by incremental increases of 0.2 °C/s up to 95 °C.

DNA Barcoding Using *rbcL*

DNA barcoding was performed using the standard method for *rbcL* proposed by the Consortium for the Barcode of Life [11]. A partial *rbcL* sequence was amplified by PCR using the primer set rbcLa_F (5′-ATGTCACCACAAA-CAGAGACTAAAGC-3′) and rbcLa_R (5′-GTAAAAT-CAAGTCCACCRCG-3′) [11]. Amplification was carried out using a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) in a total volume of 20 µL. Each reaction contained 200 µM dNTPs, 0.01 % BSA, 0.2 µM each primer, 0.1 U Ex*Taq* DNA polymerase (Takara, Otsu, Japan), 1× Ex*Taq* reaction buffer, and template DNA. Serial fivefold dilutions of *A. thaliana* genomic DNA or 2 µL of the same DNA extract employed for qPCR were used as the template DNA for DNA barcoding. The PCR conditions were as follows: 95 °C for 4 min, then 35 cycles of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s, with a final extension at 72 °C for 10 min. The amplified products were purified with the QuickStep2 PCR Purification Kit (Edge BioSystems, Gaithersburg, MD, USA).

Cycle sequencing reactions were carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Foster City, CA, USA) with a MyCycler thermal cycler in a total volume of 20 µL. Direct sequencing of the PCR products was performed using both forward and reverse primers. The cycle sequencing products were analyzed with the POP-6 Performance Optimized Polymer (Life Technologies) and detected by a 310 Genetic Analyzer (Life Technologies). The partial *rbcL* sequence was determined for each sample.

To confirm that the obtained sequences were derived from the samples, homology searches were performed for similar sequences lodged in the GenBank, European Molecular Biology Laboratory, and DNA Data Bank of Japan databases using the Basic Local Alignment Search Tool (BLAST) accessible through the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

### Development of Quantitative Real-Time PCR Method Using a Model Species

To develop a qPCR assay applicable for a wide variety of botanical samples, the primers that had successfully amplified an *rbcL* fragment in 800 plant species in Japan [38] were used for qPCR based on SYBR Green I chemistry. The PCR amplicon, the same region used for qPCR (316 bp), was purified for the generation of a standard curve. Serial tenfold dilutions of the PCR amplicon were prepared to cover the range from 10^3 to 10^7 chloroplast copies. The standard curve showed a coefficient of determination (R^2) of about 0.99 (range 0.9888–0.997), a slope of about −4.5 (range −4.21 to −4.57), and an amplification efficiency of about 65 % (range 65–67 %) ($n = 3$) (Fig. 1a). Melting curve analysis identified a single melting temperature (Fig. 1b), indicating that amplification was target specific.

To examine whether qPCR could be carried out using genomic DNA of botanical samples, qPCR was performed using serial dilutions of *A. thaliana* genomic DNA (Table 1). The triplex assay could detect 1.6 pg to 1 ng DNA. Although more dilute DNA solutions could be detected in some experiments, 1.6 pg was successfully detected in all experiments. The melting curve analysis identified a single melting temperature of about 87 °C (Table 1), indicating that amplification was target specific.

These results indicated the high reliability of the standard curve. The real-time PCR method required only 1.6 pg of *A. thaliana* genomic DNA, and thus was suitable for analysis of samples that contained an extremely small quantity of DNA.

Quantification of DNA Extracted from Common Plant Species Widely Distributed in Japan by qPCR

To examine whether quantification by real-time PCR is applicable for general analysis of botanical samples, we next quantified DNA extracts from additional plant species by qPCR. Species representing 13 plant families that are common in Japan (samples 1–15) were selected for analysis (Table 2) ($n = 3$). A standard curve was generated from the PCR amplicons, and the C_t value obtained was
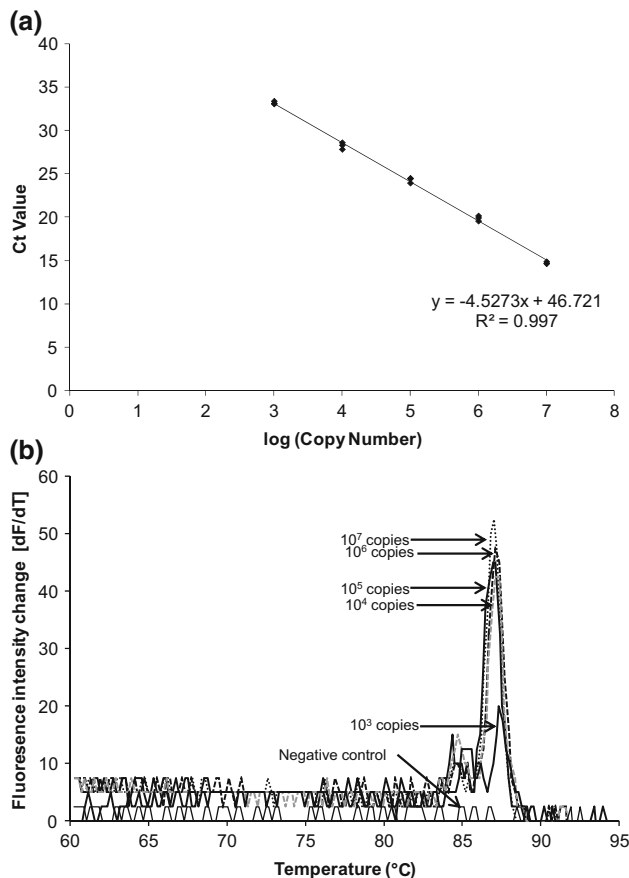
Fig. 1 Standard curve (**a**) and amplification curves (**b**) generated by qPCR using a 316-bp *rbc*L fragment as the amplicon

converted to copy numbers. The results are summarized in Table 2. Samples were diluted and two samples could not be quantified using UV–Vis methods, whereas all were quantified successfully using qPCR. Melting curve analysis identified a single melting temperature of about 87 °C, indicating that amplification was specific.

Not all samples showed a good relationship between qPCR and UV–Vis estimations. For example, although the

quantitative estimate by UV–Vis was low, qPCR analysis indicated that the DNA extract for *Rubus crataegifolius* contained a high number of chloroplast genome copies.

These results showed that the qPCR method used in this study was able to quantify DNA from a wide variety of plant species that could not be quantified by the UV–Vis method. In addition, quantifications estimated by qPCR and UV–Vis were not comparable for all species, and the two methods showed dissimilar trends among related species.

DNA Barcoding of Botanical Samples Selected for qPCR

To examine whether qPCR was able to provide information for optimization of downstream reactions, we next performed DNA barcoding using the samples selected for qPCR. The results of DNA barcoding using *A. thaliana* are shown in Table 1. All samples that were quantified by qPCR were analyzed successfully. Subsequently, DNA barcoding was performed for common representatives of 13 plant families selected for qPCR (Table 2). All samples were analyzed successfully and homology searches confirmed that the obtained sequences were derived from the samples and not from contaminants. The copy numbers were within the range of serial fivefold dilutions of *A. thaliana*. These results indicated that when the extract DNA concentration was low and a standard curve could not be obtained by UV–Vis, the amount of sample needed for DNA barcoding could be estimated from the qPCR standard curve generated for other samples.

Discussion

In the present study, a qPCR method for plant-specific absolute quantification of DNA was developed to analyze samples containing low amounts of DNA. In addition, it

Table 1 Real-time quantitative PCR (qPCR) and Ultraviolet–Visible spectrophotometry quantification of fivefold dilutions of *A. thaliana* genomic DNA and success of DNA barcoding

Quantification by UV–Visible spectrometry ng	Quantification by qPCR		DNA barcoding
	Copy numbers (average ± SD)	Tm (average)	
1.6	11,630 ± 2110	87.1	Success
8.0	61,200 ± 3110	87.1	Success
40.0	191,200 ± 44,360	87.3	Success
200.0	648,000 ± 48,580	87.2	Success
1000.0	2,480,000 ± 180,230	87.1	Success

[a] + success of DNA barcoding

Tm Melting temperature

Table 2 Real-time quantitative PCR (qPCR) and Ultraviolet–Visible spectrophotometric quantification of DNA extracted from representatives of 13 plant families common in Japan

Sample number	Family	Genus	Species	English vernacular name	Quantification by qPCR		Quantification by UV–Visible spectrophotometry	DNA barcoding	
					Copy numbers (average ± standard deviation)	Tm (average)	ng/μL	Copy Numbers used for DNA barcoding	Results of DNA barcoding
1	Asteraceae	*Cirsium*	*buergeri*	Blady grass	196,800 ± 21,760	86.9	10.5	393,605	"+[a,]"
2	Poaceae	*Microstegium*	*vimineum*	Nepalese browntop	31,700 ± 11,750	86.3	2.7	63,401	+
3	Cyperaceae	*Cyperus*	*microiria*	Asian flatsedge	13,020 ± 542	86.1	3.0	26,035	+
4	Rosaceae	*Rubus*	*crataegifolius*	Korean raspberry	392,000 ± 279,400	87.0	2.7	784,738	+
5	Rosaceae	*Rubus*	*sieboldii*	Palmleaf dewberry	132,900 ± 1205	86.1	6.6	265,794	+
6	Rosaceae	*Cerasus*	*jamasakura* (Siebold ex Koidz.)	Japanese mountain cherry	322,900 ± 17,560	85.1	8.2	645,733	+
7	Ranunculaceae	*Clematis*	*apiifolia* DC. var. *biternata*	–	170,400 ± 39,200	86.2	7.6	340,783	+
8	Lilium	*Hosta*	*sieboldiana*	Plantain lily	321,200 ± 8560	87.0	6.5	642,457	+
9	Orchidaceae	*Calanthe*	*discolor*	–	36,800 ± 14,760	86.8	–	73,512	+
10	Polygonaceae	*Persicaria*	*longiseta*	Creeping Smartweed	198,400 ± 50,860	87.1	0.2	396,900	+
11	Lamiaceae	*Scutellaria*	*indica*	–	43,400 ± 6810	87.3	2.1	86,701	+
12	Apiaceae	*Angelica*	*decursiva*	–	776,500 ± 168,590	88.0	2.3	1,553,041	+
13	Phrymaceae	*Mazus*	*pumilus*	Japanese mazus	190,800 ± 67,980	86.7	2.5	381,629	+
14	Brassicaceae	*Cardamine*	*flexuosa*	Bittercress	419,900 ± 28,590	87.3	7.3	839,703	+
15	Caryophyllaceae	*Spergula*	*arvensis*	Corn spurry	41,700 ± 11,850	87.4	–	83,479	+

+ success of DNA barcoding

[a] + success of DNA barcoding

Tm Melting temperature

was shown that the qPCR method was suitable for determination of the amount of DNA required for subsequent DNA barcoding analysis.

A relative quantification method has been reported previously to compare chloroplast gene expression levels with those of nuclear genes [37]. However, this method is not appropriate to determine the exact amount of DNA in an extract required for additional analysis of a minimum quantity of DNA, thus an absolute quantification method was developed in the present study. It was assumed that only a trace amount of DNA can be usually extracted from damaged samples and a standard curve cannot be generated for the samples. Thus, we used a PCR amplicon for a chloroplast gene to generate a standard curve. It is generally considered that the PCR amplicon used for the standard curve construction provides an accurate and reproducible quantification technique [24]. In the present study, the R^2 value of the standard curve was about 0.99, suggesting high confidence in the goodness-of-fit. Amplification efficiency was relatively low (65 %) and may have been caused by the relatively long amplicon size (316 bp) or reagent conditions, for example. DNA was quantified using an absolute quantification method in this study, and therefore the results were not influenced by the amplification efficiency.

Common plant species may have a higher chance of contributing to botanical evidence in a criminal investigation as well as in an archeological excavation because of their wide distribution or occupation of a wide variety of habitats. Therefore, common representatives of 13 plant families were selected for qPCR. The qPCR method quantified extracted DNA from all of the selected species, including the diluted or damaged samples that could not be quantified by the UV–Vis method (Table 2). This indicates the effectiveness of qPCR. However, the estimations with qPCR and UV–Vis were inconsistent. Various factors, such as the influence of nonspecific background noise for UV–Vis and the high number of chloroplast genome copies for qPCR, might have influenced the results. Copy numbers estimated by qPCR reflect the actual chloroplast genome copy number, therefore qPCR provides more reliable information for experiments that use chloroplast DNA. Additionally, use of plant-specific primer sets in qPCR allows sensitive quantification. Consequently, the qPCR method provides a means of accurately quantifying DNA in botanical samples even when contaminated with human or other animal DNA.

In the present study, the results of qPCR assisted in the decision-making will determine the success or failure of DNA analysis (Tables 1 and 2). These results indicated that when the sample DNA concentration was low and a standard curve could not be constructed for the sample, the amount of DNA needed for DNA barcoding could be estimated from the qPCR standard curve generated for other samples.

For the qPCR assay, we used SYBR Green I, which binds to the minor groove of DNA and is the most commonly used intercalating dye in modern real-time PCR systems [40]. Compared with other real-time PCR systems, such as TaqMan assays, real-time PCR using SYBR Green I chemistry is relatively flexible and inexpensive [41]. In the present study, we used an amplified fragment of 316 bp to estimate the actual amount of DNA that can be used for DNA analysis in forensic science. However, if a longer region of DNA is used for downstream reactions, it is possible to quantify samples using suitable primers for that purpose.

In conclusion, we demonstrated that qPCR analysis is an effective method with which to quantify DNA extracted from botanical samples. Moreover, this study raises the possibility that qPCR is suitable for accurate quantification of chloroplast genome copies from a wide variety of plant species and from diluted or damaged samples. The results of qPCR analysis and DNA barcoding showed strong comparability, indicating the possibility of optimization of the procedure for downstream reactions.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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