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ORIGINAL ARTICLE

Quantitative evaluation of properties of residual DNA in *Cryptomeria japonica* wood

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Abstract Genomic DNA sequences code important information that enables the identification of organisms at the species level. Investigating the properties of residual DNA in wood can significantly contribute to the widespread and effective application of the DNA identification of wood materials and wood products. In this study, residual DNA was extracted from the sapwood and heartwood of *Cryptomeria japonica* with different elapsed years after cutting, and their properties were estimated by quantitative polymerase chain reaction (qPCR) analyses. There was no practical correlation between the amount of residual DNA extracted and the copy numbers of plastid DNA fragments, suggesting that the amount of extracted residual DNA was not an important requirement for the successful DNA identification of wood species. In the sapwood, the amount of residual plastid DNA with sufficient length for PCR amplification gradually decreased in line with more elapsed years after cutting, whereas in the heartwood that was not clearly correlated with the elapsed years after cutting. It was considered that both sapwood and heartwood were effective for the DNA identification of wood products and constructions. The plastid DNA fragments contained in wood material cut more than 400 years ago could be extracted and amplified, and their copy numbers evaluated. The results obtained suggested that targeting and amplifying a plastid DNA fragment with a length of approximate 100 bp enable the analysis of the genetic background of wood materials used in the historically important products and constructions.

Keywords DNA · Plastid · qPCR · Wood identification

Introduction

The accurate identification of wood species is extremely important for the taxonomy of woody plants. Such identification can also provide key evidence regarding the quality assurance of wood products, exposure of illegal logging, and authentication of historically important wood products and architecture. The traditional method of identifying wood species entails using a microscope to observe the anatomical structure, in which wood specimens are identified based on anatomical characteristics such as the shape and arrangement of secondary xylem cells, the structure and components of ray tissue, the size and arrangement of pits, and other aspects [1, 2]. Microscopic observation enables the identification of wood species at the genus level, but judgment at the species level is limited in this method [3, 4].

The genomic DNA in nuclei and organelles includes the base sequences inherent in biological species. These sequences are frequently investigated to identify living organisms at the genus and species levels. The extraction and polymerase chain reaction (PCR) amplification of residual DNA in wood have been attempted to identify

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wood species, and their effectiveness have been exhibited [3–13]. For the identification of wood species, each specimen is subjected to different conditions such as elapsed years after cutting, processing, usage, and other conditions that may affect the quality and quantity of residual DNA. The residual DNA in old wood products and constructions is considered to be damaged physically or chemically compared with plant samples kept under anaerobic conditions such as in sediment or in water. In order to apply the DNA identification of wood materials more generally and effectively, the influences of the storage period after harvesting wood samples on the properties of residual DNA should be quantitatively clarified. This information is expected to be useful, especially for identifying the wood used as the materials of historically valuable products and buildings.

In this study, residual DNA was extracted from the sapwood and heartwood with different elapsed years after cutting, and their properties were investigated by quantitative PCR (qPCR) analyses. Wood specimens collected from members of a historical structure were used as old wood materials. From the results obtained, variations in the properties of residual DNA in wood were evaluated, and the effectiveness of residual DNA in accurately identifying old wood materials was discussed.

Materials and methods

Wood materials

The sapwood and heartwood of *Cryptomeria japonica* D. Don were used in this study. All materials examined are from the collections of xylariums of the Forestry and Forest Products Research Institute and Kyoto University. Table 1 lists the material names, parts, ages of trees, elapsed years after cutting, basic densities and the Wood ID nos. for all materials. The materials TWTw6429 and TWTw96 had been used to extract residual DNA in the previous report [3]. The material KYOw17215 was collected from the sheathing roof boards of the Kitasomon gate at Toji Temple in Kyoto, Japan. These materials were presumed as having been cut and processed before the Edo-Keicho period (1596–1614) according to a tradition in the temple.

Preparation of wood powder and extraction of residual DNA

Small pieces of 1 mm by 1 mm by 5 mm were prepared from each material listed in Table 1. After being precooled in liquid nitrogen, the samples were powdered with a disruptor (Multi-Beads Shocker, Yasui Kikai). The revolution speed was 2500 rpm for a milling time of 30 s. All wood powder samples were prepared in 2010, and then stored at temperatures below −80 °C just prior to the extraction of residual DNA.

The residual DNA contained in 500 mg of each wood powder was extracted using the DNeasy Plant Maxi Kit and Mini Kit (Qiagen), and then finally eluted in 50 μl of ultra pure water. The extracting operation was conducted three times for each wood powder sample. The concentrations of DNA extracted were measured using a fluorometer (Qubit 2.0, Life Technologies Co.).

Quantitative PCR analysis of residual DNA in wood

The lengths and copy numbers of the genes encoding the large sub-unit of ribulose 1,5-bisphosphate carboxylase/oxygenase (rbcL) of *C. japonica* (accession No. L25751) in the extracted residual DNA were investigated by qPCR analyses. Table 2 lists the amplicon lengths, sense and antisense primer sequences, and TaqMan® probe sequences

Table 1 Wood materials for extraction of residual DNA

Material name	Part	Age of tree	Elapsed years after cutting	Basic density (g/cm^3)	Wood ID no.
0-S	Sapwood	12	0	0.41	–
0-H	Heartwood	12	0	0.38	–
4-S	Sapwood	23	4	0.30	–
4-H	Heartwood	23	4	0.32	–
14-S	Sapwood	43	14	0.26	TWTw15981
14-H	Heartwood	43	14	0.27	TWTw15981
27-S	Sapwood	Unknown	27	0.37	TWTw6429
27-H	Heartwood	Unknown	27	0.36	TWTw6429
44-S	Sapwood	Unknown	44	0.33	TWTw96
44-H	Heartwood	Unknown	44	0.38	TWTw96
400-S	Sapwood	Unknown	More than 400 years	0.21	KYOw17215
400-H	Heartwood	Unknown	More than 400 years	0.30	KYOw17215

No Wood ID nos. are provided for 0-S, 0-H, 4-S and 4-H

(Dual-labeled probe and primers for QPCR, Sigma Aldrich Japan). The copy numbers of *rbcL* fragments were estimated using a standard curve method. A 364 bp fragment of *C. japonica rbcL* (nucleotide positions 363–726) was amplified using the TaKaRa ExTaq™ HotStart version (Takara Bio Inc.) as a PCR enzyme and using Ampdirect® Plus (Shimadzu Corporation) as a buffer solution. A 1:10 dilution series was prepared using this PCR product. The initial copy numbers of 1:10 dilution series were 2.00×10^6 copies per µl for the 93 and 185 bp amplifications and 2.00×10^7 copies per µl for the 317 bp amplification, respectively. The qPCR analyses were carried out in a StepOne™ system (Applied Biosystems) programmed for: 1 cycle at 50 °C for 2 min, then 95 °C for 10 min; followed by 40 cycles each consisting of 15 s at 95 °C and 1 min at 60 °C. The reaction solutions contained 5 % extracted DNA (volume), 2.7 µM sense and anti-sense primers, 0.68 µM probe, the TaqMan® Gene Expression Master Mix (Applied Biosystems) and ultra pure water. The correlation coefficients of the standard curves in this experiment were very high ($r^2 = 0.99$), indicating that the primers and probes listed in Table 2 precisely functioned. The PCR efficiency of each measurement was determined from the amplification curves of the dilution series obtained. Experimental data with 80–100 % of the efficiencies were used to estimate the copy numbers of *rbcL* in the extracted residual DNA.

Microscopic observation

The states of nuclei, amyloplasts, and other organelles in the materials examined in this study were observed by fluorescence light microscopy. Small blocks with dimensions of 5 mm (longitudinal) by 5 mm (radial) by 5 mm (tangential) were collected from each material with a fresh razor blade, and then fixed in 3 % glutaraldehyde. After dehydration in a graded series of ethanol and the substitution with propylene oxide, the blocks were embedded with LR-White resin (London Resin Co.). Radial sections 2 µm thick were cut from the embedded blocks by a rotary microtome (HM-340E, Microm), and then mounted on grass slides. The sections were stained with 0.2 % 4′,6-diamidino-2-phenylindole (DAPI). These sections were then observed under a microscope (BX-41, Olympus).

Results and discussion

Amount of residual DNA extracted and copy numbers of *rbcL* fragments

Figure 1 shows the relationship between the elapsed years after cutting and the amount of total residual DNA

Table 2 Sequences of sense primers, anti-sense primers and probes for the quantitative PCR analyses

Amplicon length (bp)	Sense primer	Anti-sense primer	Probe
93	GAAAACGTGAATTCCCAACCATT (nucleotide positions 580–602)	CTCACCCGTCTCAGCCTGA (nucleotide positions 672–654)	ACAAAAGCAGAAACGATCTCTCCAGCGC (nucleotide positions 633–606)
185	CCAAGGCCCACCACATGG (nucleotide positions 414–431)	GTTGGGAATTCACGTTTTCATCATC (nucleotide positions 598–574)	TCCACCACGGAGACATTCATAAACCGCT (nucleotide positions 558–531)
317	CGGATTCCTCCTGCTTATTCAAAA (nucleotide positions 385–408)	GCAGTAGCATTCAGGTAATGTCC (nucleotide positions 701–679)	TCCAAGGCCCACCACATGGTATTCAAGT (nucleotide positions 413–440)

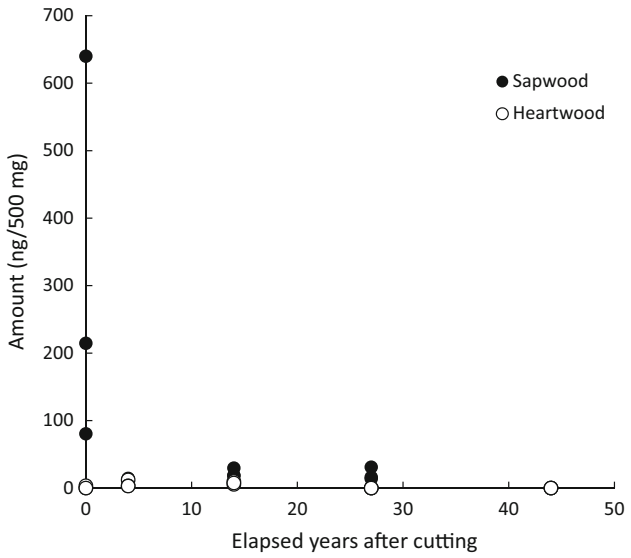

Fig. 1 Relationship between elapsed years after cutting and amount of total residual DNA extracted

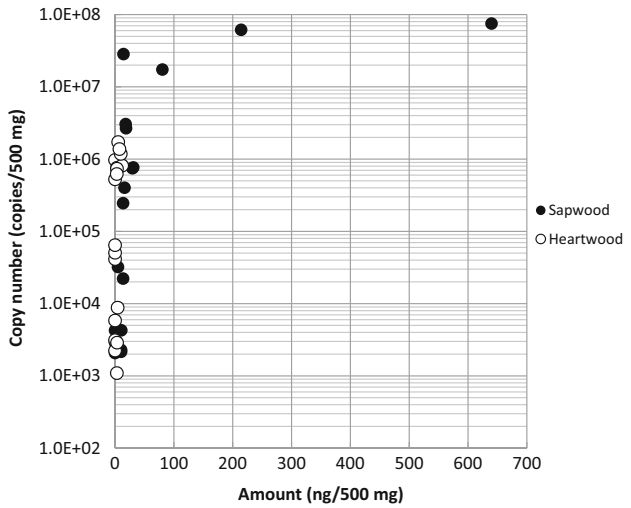

Fig. 2 Relationship between amount of total residual DNA extracted and copy numbers of 93 bp *rbcL* fragments

extracted for all the materials examined. The yields of residual DNA measured in this study were smaller than the results previously reported [6, 14]. The amounts for 0-S were significantly larger and ranged from 80.5 to 640 ng/500 mg. Except for 0-S, the amounts of residual DNA extracted from the sapwood were below 35.0 ng/500 mg. It was impossible to measure the amount for 44-S. The amounts of residual DNA extracted from the heartwood were below 15.0 ng/500 mg. The amounts for 27-H and 44-H could not be measured. During the withering process for *C. japonica*, the volume of the nuclei in ray parenchyma cells decreased, and the nuclei in early wood regions finally disappeared 150 days after cutting [15]. The genomes of conifers are the largest among all organisms, typically 20 to 30 Gbp [16, 17]. The chloroplast genome of *C. japonica* has 131810 bp [18], which constitutes only a small amount of the total genome. From these findings, it was considered that the much lower amount of residual DNA for 4-S than that for 0-S was attributed to the degradation and disappearance of the nuclei in ray parenchyma cells in early wood regions 4 years after cutting. Because the nuclei in ray parenchyma cells of heartwood had already disappeared before heartwood formation, an extremely small amount of residual DNA might remain in the heartwood regardless of the age of tree or the elapsed years after cutting.

Figure 2 shows the relationship between the amounts of residual DNA and the copy numbers of 93 bp *rbcL* fragments. Below the amount of 40 ng/500 mg, copy numbers largely varied and differed by a factor of more than 1000. There was no clear difference in copy numbers between sapwood and heartwood materials at the low amount of residual DNA extracted.

Since the copy numbers were large for 0-S, a positive correlation was recognized between the amounts of residual DNA and the copy numbers of *rbcL* fragments ($R^2 = 0.80$). However, the copy numbers obtained varied significantly at the low amount of DNA, so that there was no practical correlation between both values. This trend was also observed in results for the 185 and 317 bp *rbcL* fragments. These results suggest that the amount of residual DNA extracted from a wood sample is not an important requirement for the successful detection of the targeted sequences by PCR.

Elapsed years after cutting and copy numbers of *rbcL* fragments

Figure 3 shows the relationship between the elapsed years after cutting and the copy numbers of 93 bp *rbcL* fragments. In the residual DNA extracted from 0-S, the copy numbers were estimated to be from 1.73×10^7 to 7.50×10^7 copies/500 mg (Fig. 3a). The copy numbers for 4-S largely varied even though the residual DNA was extracted from the same wood powder. The copy numbers for 44-S were estimated to be approximately one ten-thousandth of those for 0-S. In the sapwood of *C. japonica*, the copy numbers of 93 bp *rbcL* fragments gradually decreased with more elapsed years after cutting except those for 4-S. In the residual DNA extracted from 0-H, the copy numbers were estimated to be from 5.25×10^5 to 9.73×10^5 copies/500 mg (Fig. 3b), and thus significantly smaller than those for 0-S. The copy numbers for 4-H and 14-H were almost at the same level as those for 0-H. The copy numbers for 27-H were estimated to be approximately one hundredth of those for

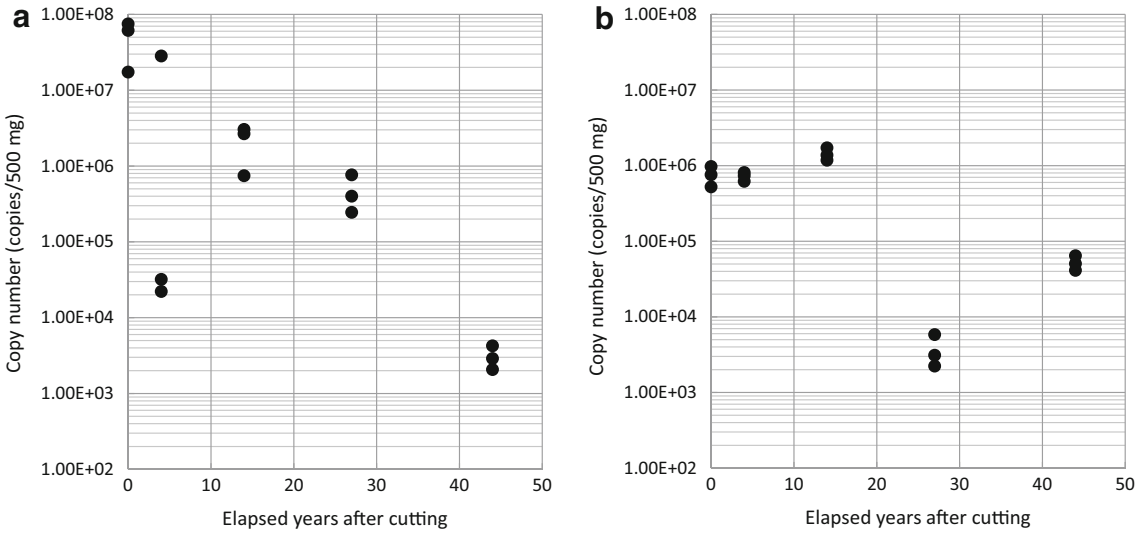

Fig. 3 Relationship between elapsed years after cutting and copy numbers of 93 bp *rbcL* fragments. **a** sapwood, **b** heartwood

0-H. The copy numbers for 44-H, however, were estimated as being approximately ten times as large as those for 27-H, even though more years had elapsed after cutting. For the heartwood of *C. japonica*, the copy numbers of 93 bp *rbcL* fragments in the residual DNA did not depend on the elapsed years after cutting. In the previous report, it was somewhat difficult to detect the 82 bp *rbcL* fragments in the residual DNA by PCR amplification and subsequent gel electrophoresis for 44-S and 27-H [3]. In this experiment, the copy numbers of 93 bp *rbcL* fragments for these materials were below 1.00×10^4 copies/500 mg. Therefore, more than 2.00×10^4 copies of DNA are sufficient number for successful DNA amplification by conventional PCR in 1 g of wood.

Figure 4 shows the relationship between the elapsed years after cutting and the copy numbers of 185 bp *rbcL* fragments. As in the results shown in Fig. 3a, the copy numbers in the residual DNA extracted from sapwood materials gradually decreased in line with more elapsed years after cutting (Fig. 4a). The copy number was undetectable for one of the three samples of residual DNA extracted from 44-S. For the heartwood, the copy numbers in the residual DNA did not depend on the elapsed years after cutting, as in the case for 93 bp amplification (Fig. 4b). The copy numbers could not be detected for the residual DNA extracted from 27-H.

Figure 5 shows the relationship between the elapsed years after cutting and the copy numbers of 317 bp *rbcL* fragments. As in the results shown in Figs. 3a and 4a, the copy numbers in the residual DNA extracted from sapwood materials gradually decreased in line with more elapsed years after cutting (Fig. 5a). The copy numbers could not be detected for the residual DNA extracted from 44-S. The copy numbers also could not be detected for the residual DNA extracted from 27-H and 44-H (Fig. 5b).

For the sapwood of *C. japonica*, the amount of residual plastid DNA having sufficient length for PCR amplification is considered to gradually decrease in line with more elapsed years after cutting. The sapwood material cut more than 40 years ago is inferred to contain quite a small number of plastid DNA fragments with lengths of more than 300 bp. Conversely, in the heartwood, the amount of residual plastid DNA having sufficient length for PCR amplification may not clearly depend on the elapsed years after cutting. The heartwood material cut more than 20 years ago is inferred to contain quite a small number of plastid DNA fragments with lengths of more than 300 bp. In the previous report [3], it was difficult to detect the 527 bp *rbcL* fragments for materials cut more than 27 years ago. Figure 5 shows results corresponding to this previous result.

Amplification length of *rbcL* fragments and copy numbers

Figure 6 shows the relationship between the DNA lengths for amplification and the copy numbers for *C. japonica* *rbcL* fragments. This figure also includes data on the residual DNA extracted from members (400-S and 400-H) of the Kitasomon gate. The 317 bp *rbcL* fragments existed in the residual DNA extracted from 0-S, 4-S, 14-S and 27-S (Fig. 6a). For the residual DNA extracted from 0-S and 14-S, the copy numbers of 317 bp *rbcL* fragments were approximately one-tenth of those of 93 bp *rbcL* fragments. The residual DNA extracted from 0-H, 4-H, and 14-H contained the 317 bp *rbcL* fragments (Fig. 6b). The

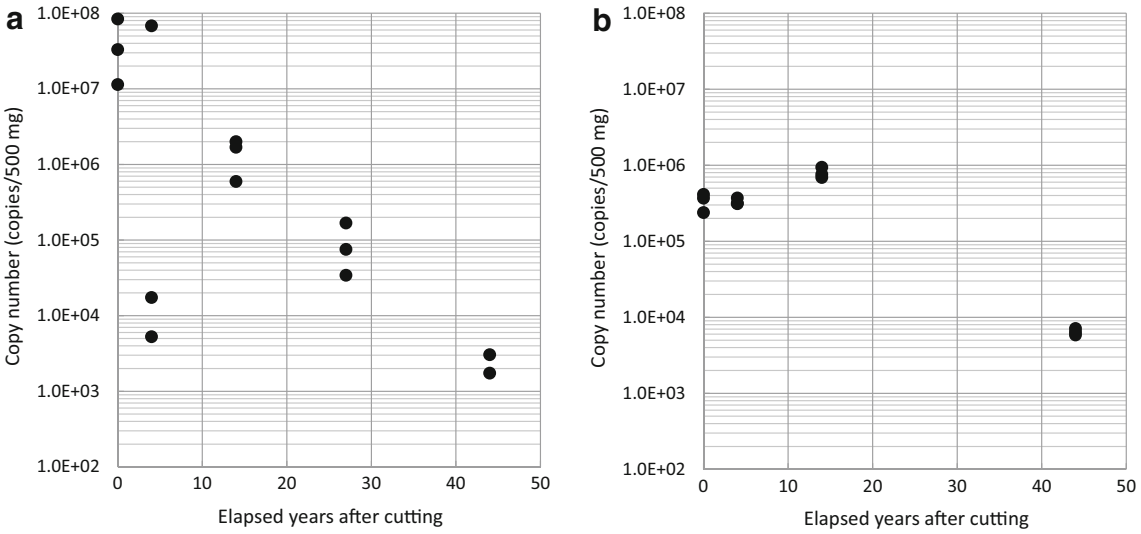

Fig. 4 Relationship between elapsed years after cutting and copy numbers of 185 bp *rbcL* fragments. **a** sapwood, **b** heartwood

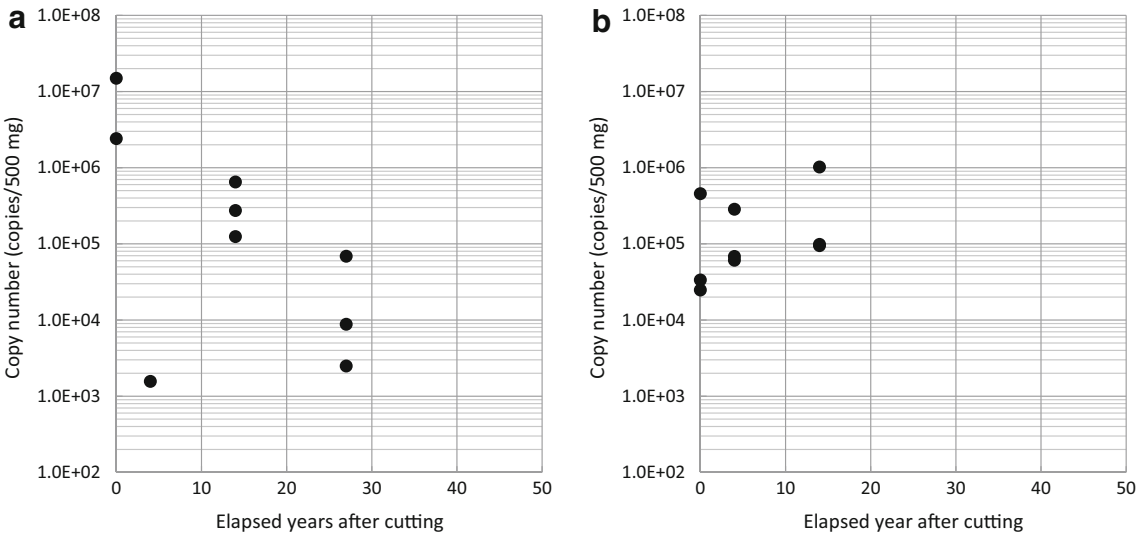

Fig. 5 Relationship between elapsed years after cutting and copy numbers of 317 bp *rbcL* fragments. **a** sapwood, **b** heartwood

residual DNA extracted from 400-S contained both the 93 and 185 bp *rbcL* fragments. The copy numbers of 93 bp *rbcL* fragments were estimated to be from 2.15×10^3 to 4.26×10^3 copies/500 mg, almost the same values as for 44-S. The 185 bp *rbcL* fragments could be amplified in one of the three residual DNA samples extracted from 400-S. The residual DNA extracted from 400-H also contained both the 93 and 185 bp *rbcL* fragments. The copy numbers of 93 bp *rbcL* fragments were estimated to be from 1.09×10^3 to 8.78×10^3 copies/500 mg, almost the same values as for 27-H. The 185 bp *rbcL* fragments could be amplified in two of the three residual DNA samples extracted from 400-H. In this experiment, the copy numbers of residual plastid DNA fragments stored in very old wood materials could be quantified. This result will be very useful for the DNA identification of old wood. The amplicon size of targeted DNA is a crucial factor because the DNA extracted from wood is partially degraded [19]. The experimental result as shown in Fig. 6 is a good guide to the effective PCR amplification of DNA fragments extracted from the wood being investigated.

Nuclei and amyloplasts in ray parenchyma cells of *C. japonica* sapwood and heartwood

Figure 7a and b shows the nuclei and amyloplasts in the ray parenchyma cells of 0-S, respectively. Almost all the nuclei observed did not retain their spherical shapes

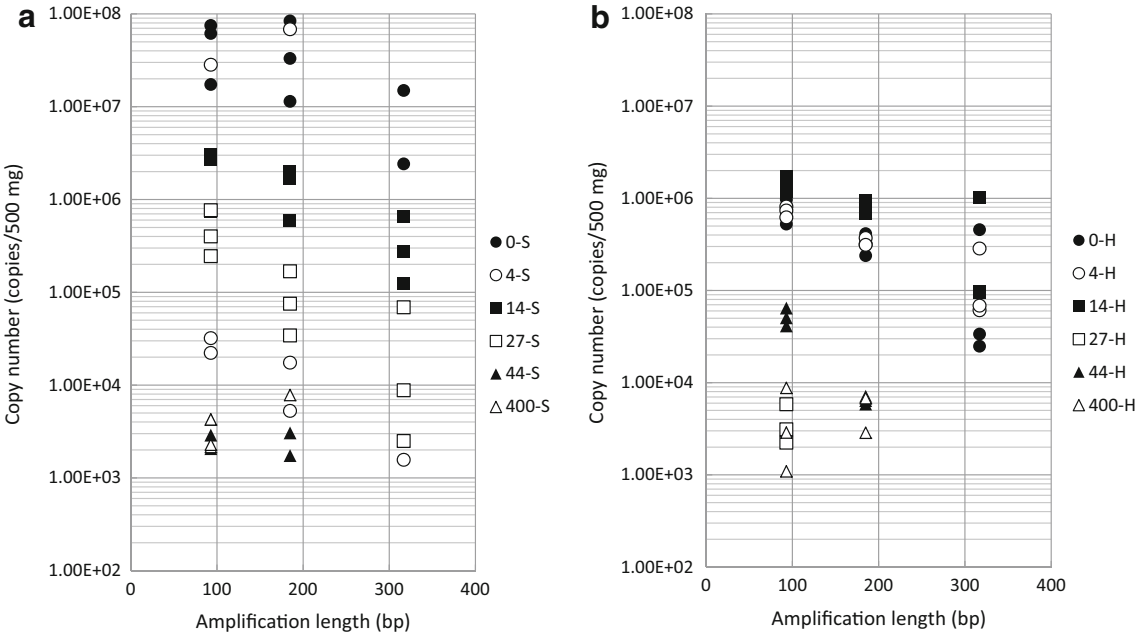

Fig. 6 Relationship between length of *rbcL* fragments (bp) and copy numbers. **a** sapwood, **b** heartwood

(Fig. 7a). There were many amyloplasts in the cells (Fig. 7b). More deformed nuclei were observed in the ray parenchyma cells of 4-S, and part of the nuclei showed yellowish brown (Fig. 7c). Although the fluorescence intensity was weak, amyloplasts were observed in the cells. It was difficult to observe both nuclei and amyloplasts in the sapwood of 44-S (Fig. 7d). These results correspond to the significant reduction in the amount of extracted DNA in the sapwood as shown in Fig. 1.

No nucleus was observed in the heartwood ray parenchyma cells (Fig. 7e, f). Only a small amount of amyloplasts remained in the ray parenchyma cells of 44-H (Fig. 7f), thereby corresponding to the copy numbers of 185 bp *rbcL* fragments obtained for 44-H as shown in Fig. 4.

Effectiveness of residual DNA for the identification of old wood material

Plastid genome markers are often chosen to identify plant species at the DNA sequence level [20]. The *rbcL* gene encoding the sub-unit of the key enzyme of the Calvin cycle is located on the plastid genome. The detection of *rbcL* fragments by PCR amplification was utilized to discriminate the orders, families or genera of plants contained in extinct animal dung [21, 22] and sediments [23]. This procedure was effective in verifying the presence of DNA in archaeological *Vitis vinifera* seeds conserved in water [24]. In addition, analyzing the spacers between transfer RNA coding segments on plastid DNA enabled the identification of plant specimens at an intraspecific level [25] as well as at the genus level [26]. As shown in Fig. 7a, ray parenchyma cells in freshly cut sapwood possessed many amyloplasts. Unlike the significant reduction in the amount of total residual DNA for sapwood (Fig. 1), the copy numbers of *rbcL* fragments gradually decreased in line with more elapsed years after cutting. The copy numbers of residual plastid genomic DNA are estimated to be larger than those of residual nucleus genomic DNA. The fragments of plastid DNA containing *rbcL* could also be extracted from *C. japonica* wood cut and processed more than 400 years ago, and their copy numbers evaluated. Therefore, targeting the sequences on plastid genomic DNA may be effective for the accurate identification of old wood materials, as with the DNA identification of plant species contained in animal dung and sediments.

The materials 0-H, 4-H, and 14-H were collected from trees that were 12, 23, and 43 years old, respectively. Among the DNA extracted from these samples, there was a small difference in the copy numbers of residual plastid DNA below the amplification length of 200 bp (Fig. 6b). These results suggest that the fragmentation of plastid DNA progresses gradually during heartwood formation within a tree, and then is suppressed after the complete transition into heartwood. The residual DNA extracted from 4-H probably contained larger copy numbers of plastid DNA than that from 4-S. A similar result was obtained for 44-H and 44-S. These results indicate that the plastid DNA in sapwood is easily fragmented as compared with that in heartwood. Therefore, it is considered that the

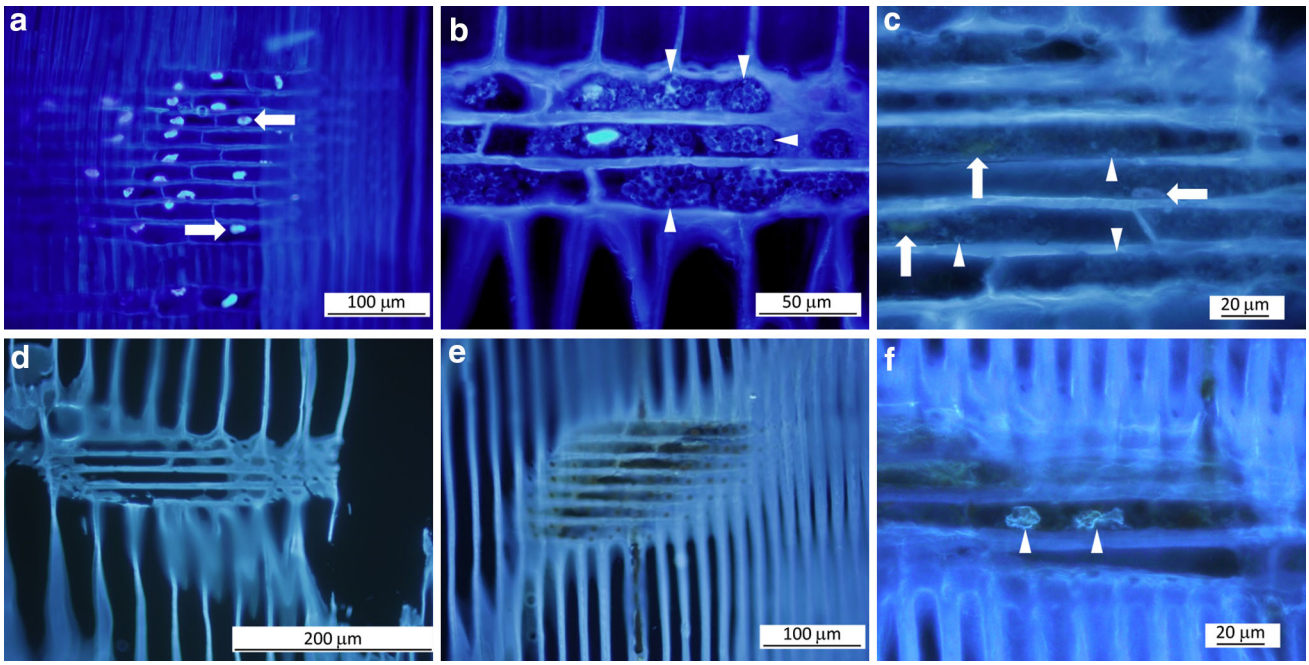

Fig. 7 Fluorescent light micrographs of *C. japonica* ray parenchyma cells. **a** and **b** 0-S, **c** 4-S, **d** 44-S, **e** 0-H, **f** 44-H. *Arrows* indicate nuclei; *arrowheads* indicate amyloplasts

copy numbers of residual plastid DNA do not depend on the part of wood, and both sapwood and heartwood are available for the DNA identification of wood products.

From the results shown in Fig. 6a and b, the amounts of residual plastid DNA in 400-S and 400-H are inferred to be roughly the same levels as in 44-S and 44-H, suggesting that residual plastid DNA in old wood materials is fragmented to a length of approximate 200 bp and stably stored in heartwood. Targeting the sequences of plastid DNA with a length of approximate 100 bp may be effective for the DNA identification of wood used in historically important products and constructions.

Conclusion

In this study, the properties of residual DNA extracted from the sapwood and heartwood of *C. japonica* with different elapsed years after cutting were investigated by using quantitative PCR analyses. There was no practical correlation between the amounts of residual DNA extracted from wood samples and the copy numbers of *rbcL* fragments. The amount of residual DNA extracted is not an important requirement for the successful DNA identification of wood species. In the sapwood, the amount of residual plastid DNA having a sufficient length for PCR amplification gradually decreased in line with more elapsed years after cutting, whereas in the heartwood it was not clearly correlated with the elapsed years after cutting. It was considered that the numbers of residual plastid DNA did not depend on the part of wood, and both sapwood and heartwood were effective for the DNA identification of wood products. The residual DNA in wood material cut more than 400 years ago could be extracted, and a plastid DNA fragment with a length of approximate 200 bp could be amplified. For identifying the wood materials used in historically important products and constructions, targeting a plastid DNA fragment with a length of approximate 100 bp ensures successful DNA amplification for DNA analysis.

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