For. Path. 30 (2000) 157–164

ISSN 1437–4781

A simple, polymerase chain reaction–restriction fragment length polymorphism-aided diagnosis method for pine wilt disease

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Summary

For diagnosis of pine wilt disease, a simple PCR-RFLP method was developed to identify and to differentiate two similar nematode species, based on a living or preserved single specimen. Pinewood nematodes, *Bursaphelenchus xylophilus*, and *Bursaphelenchus mucronatus* were examined. A single nematode in 1 μl of distilled water was put on a glass slide. When the water had almost dried the nematode was crushed with a filter paper chip, 1.5 mm × 1.5 mm, with the aid of forceps. The filter paper chip containing nematode remains was immediately placed into PCR buffer as the DNA template. The primer set used was to amplify ribosomal DNA containing the inter-transcribed spacer (ITS) 1, 5.8S and ITS2 regions. The PCR product was consistently obtained from a single nematode, and digesting the product with restriction endonuclease, *Hin*f I, enabled discrimination between *B. xylophilus* and *B. mucronatus*. This method was simple, convenient and definitive, and could successfully determine the pathogen in the diagnosis of pine wilt disease. This method was applicable also to nematode specimens preserved under various conditions except in the case of those preserved in aldehyde-containing fixatives.

1 Introduction

In Japan, pine wilt disease caused by pinewood nematode (*Bursaphelenchus xylophilus*) has been a serious problem. A mucro on the tail tip of the adult female distinguishes *Bursaphelenchus mucronatus*, a closely related nonpathogenic species, from *B. xylophilus* and has been regarded as a determinative character (MAMIYA and ENDA 1979). However, a mucro-like structure was often observed also on adult female *B. xylophilus*, although it is somewhat shorter than that of *B. mucronatus*. Furthermore, male adults or juveniles cannot be used for identification of these two species by this method. Therefore, the discrimination between these two species is sometimes difficult. An alternative method which is simple, convenient, and definitive is urgently needed in order to determine with certainty the pathogen associated with pine wilt disease.

Many attempts have been made for nematode identification or phylogenetic studies using DNA-based methods, such as restriction fragment length polymorphism (RFLP) (NASMITH et al. 1996; ORUI 1996; IWAHORI et al. 1998), random-amplified polymorphic DNA (RAPD) (BLOK et al. 1997; THIÉRY et al. 1997), amplified fragment length polymorphism (AFLP) (PETERSEN and VRAIN 1996; VAN DER BEEK et al. 1998), and DNA sequencing (ADAMS et al. 1998; DE BLAXTER et al. 1998). These techniques provide the basic methodology that is needed for such a diagnostic test.

This paper reports on a simple, modified polymerase chain reaction-restriction fragment

Received: 11.10.1999; accepted: 25.2.2000; editor: J. Stenlid

length polymorphism (PCR-RFLP) method using an egg, a second-stage juvenile, or an adult of living *B. xylophilus* to provide an unambiguous identification. This method was also applied to nematode specimens that had been stored in common preservatives, and from field nematode samples extracted from a pine tree showing symptoms of pine wilt.

2 Materials and methods

2.1 Nematodes

Bursaphelenchus xylophilus isolate S10 (originally isolated from Shimane Pref., Japan) and *B. mucronatus* isolate M (from Kyoto Pref., Japan) were reared on a fungal mat of *Botrytis cinerea* grown on autoclaved barley at 20°C for about a month, then collected by modified Baermann funnel method (IWAHORI and FUTAI 1993) before use. Nematodes from field samples were separated from wood chips of a wilted Japanese red pine tree (*Pinus densiflora*) sampled in Osaka Pref., Japan in autumn 1997.

2.2 Nematode preserving

Cultured nematodes were preserved by five methods as follows: Fr; suspended in distilled water and frozen at −20°C; or suspended in E, 99.5%(v/v) ethanol; G, 3%(v/v) glutaraldehyde; Fo, 10%(v/v) formalin; and T, TAF (7%(v/v) formalin, 2%(v/v) triethanolamine). Treatments E, G, Fo and T were stored at room temperature. Preserving periods tested were 1 week, 1 month and 3 months.

2.3 DNA extraction from a single nematode

The eggs, the second-stage juveniles, and the adult nematodes were picked up with a sharpened-tip wire. In the case of preserved specimens, they were immersed in distilled water for about 30 min to wash out fixatives and then picked up. A single nematode sample was transferred into a 1 μl drop of distilled water on a glass slide (Fig. 1). As soon as the

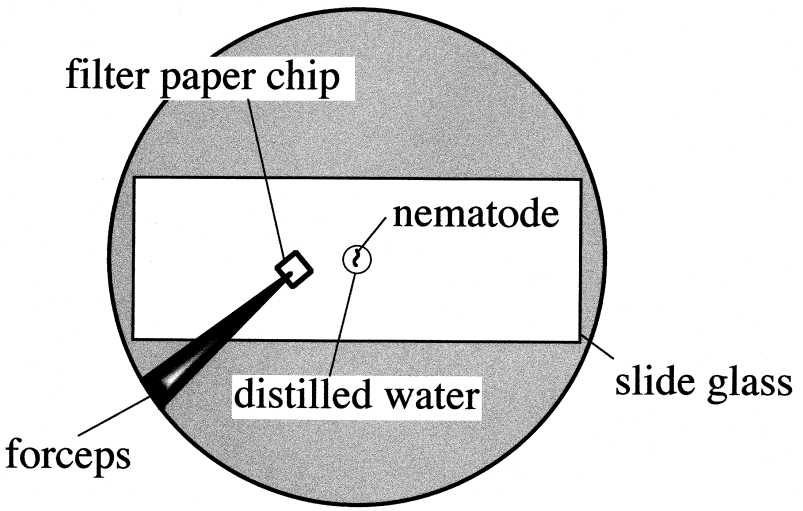

Fig. 1. Procedure for crushing a nematode

water dried, the nematode specimen was crushed with a filter paper chip (no. 4 A; Toyo Roshi Co., Ltd., Tokyo, Japan), approximately 1.5 mm × 1.5 mm, with the aid of forceps under the stereoscopic microscope. The filter paper chip, with nematode remains as DNA template, was immediately placed into a 0.2 ml micro test tube containing 25 μl of PCR solution (10 μM of each primer, 1.3 μl; 2.5 mM of dNTP, 4 μl; 5 U/μl of Taq polymerase, 0.2 μl; 10 × reaction buffer, 2.5 μl; autoclaved ultra pure distilled water, 15.7 μl) and mixed well. In the case of preserved specimens the DNA template also was prepared from five nematodes, and the procedure was the same as for the single fresh nematode specimen.

2.4 PCR amplification

The primers were selected to amplify ribosomal DNA containing the internal transcribed spacer 1 (ITS1), 5.8S rDNA, and ITS2 regions of a total length of about 0.88 kb. The sequence of the forward primer, 5′-CGTAACAAGGTAGCTGTAG-3′, and that of the reverse primer, 5′-TCCTCCGCTAAATGATATG-3′, were derived from the data of FERRIS et al. (1993). The amplification was carried out with a thermocycler (TaKaRa PCR Thermal Cycler TP-240; TaKaRa Co. Ltd., Tokyo, Japan) and reaction conditions were as follows: at 94°C for 2 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 49°C for 15 s, polymerization at 72°C for 1 min, and a final extension cycle at 72°C for 10 min. After PCR was completed, 5 μl of amplified product from each sample with 1 μl of × 6 BPB dye marker solution (0.25%(w/v) bromophenol blue, 30%(v/v) glycerol) were analysed by electrophoresis in a 1.2%(w/v) agarose gel in TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 2 mM EDTA, pH 8.1) for 30 min at 100 V. The gel was stained with 2.5 mg/l ethidium bromide for 15 min, photographed under UV light, and the generation of the PCR product was checked.

2.5 Restriction enzyme treatment

Four micro litres of the PCR product, 0.5 μl (10 U) of *Hin*f I and 0.5 μl of 10 × reaction buffer were mixed well and incubated at 37°C overnight to digest the PCR product completely. This restriction enzyme clearly discriminates between *B. xylophilus* and *B. mucronatus* but does not show any difference in RFLP among isolates of each species (IWAHORI et al. 1998). The DNA fragments thus generated were separated by electrophoresis with 1 μl of × 6 BPB dye marker solution in a 6%(w/v) polyacrylamide gel in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) for 80 min at 100 V. The gel was stained with ethidium bromide, visualized, and photographed under UV light in the same way as described above. The resulting RFLP patterns were used to discriminate between the two species.

3 Results

The method of crushing an individual nematode with a filter paper chip was eminently successful and a useful method to obtain a DNA template of nematode for PCR. Only one common PCR product was constantly obtained from a crushed single egg, a second-stage juvenile, or an adult nematode of *B. xylophilus* and *B. mucronatus* (Fig. 2a). The size of this fragment was about 872 bp and was the same as that evaluated for the DNA template extracted from bulk individuals of *B. xylophilus* or *B. mucronatus* (IWAHORI et al. 1998). Further, digestion of the PCR product with restriction endonuclease, *Hin*f I, enabled the definitive discrimination of *B. xylophilus* from *B. mucronatus* (Fig. 2b).

To examine the applicability of this method for diagnosis of pine wilt, 10 nematodes each of either fourth-stage juvenile or adult were sampled at random from wood chips of a

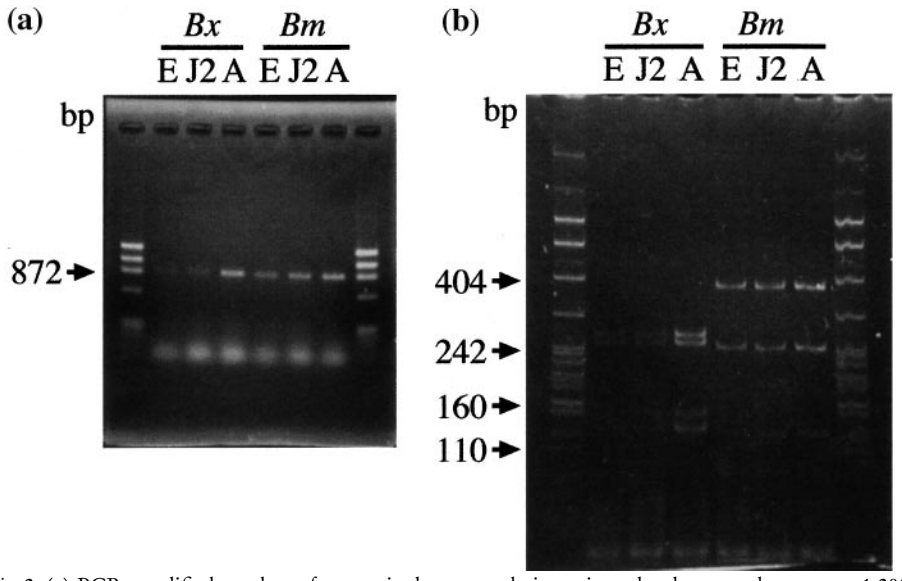

Fig. 2. (a) PCR-amplified products from a single nematode in various developmental stages on 1.2% agarose gel, and (b) *Hin*f I digested-PCR-RFLP patterns on 6% polyacrylamide gel: *Bx*, *Bursaphelenchus xylophilus*; *Bm*, *B. mucronatus*; E, egg; J2, second-stage juvenile; A, adult. Molecular weight markers are shown in base pairs (bp)

diseased pine tree in the field and crushed individually to obtain DNA samples. All samples served for PCR gave a single band of approximately 872 bp (Fig. 3a). *Hin*f I digestion of PCR products produced the same RFLP pattern as *B. xylophilus*, not as *B. mucronatus* (Fig. 3b). Therefore, the field samples of nematodes examined here were identified as *B. xylophilus,* and the disease could be diagnosed as being caused by pinewood nematode.

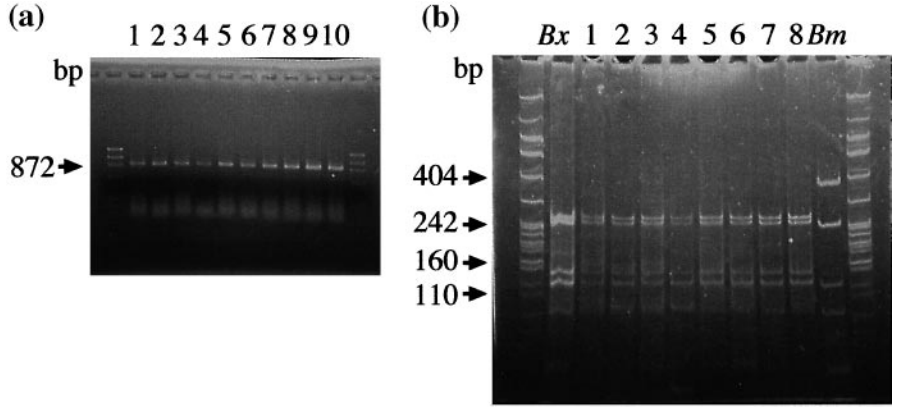

Fig. 3. (a) PCR-amplified products from field samples on 1.2% agarose gel, and (b) *Hin*f I-digested PCR-RFLP patterns on 6% polyacrylamide gel: *Bx*, *Bursaphelenchus xylophilus*; *Bm*, *B. mucronatus*. Numbers 1–10 are individual samples and numbers 1–8 correspond in both (a) and (b). Molecular weight markers are shown in base pairs (bp)

Figure 4a shows the PCR-amplification products of a 1-week-preserved specimen of single or five *B. xylophilus* and *B. mucronatus* individuals. The PCR products were successfully obtained from both nematode species preserved in distilled water at −20°C or in 99.5% ethanol at room temperature. These preserved specimens of both *Bursaphelenchus* species could be discriminated from each other by digesting PCR products with *Hin*f I (Fig. 4b). On the other hand, PCR products were not generated from specimens preserved in 3% glutaraldehyde, 10% formalin or in TAF.

The results obtained from 1-month- or 3-month-preserved specimens were mostly the same as those mentioned above for 1-week-preserved specimens (Fig. 5a,b). However, sometimes unexpected bands of a size less than 872 bp were observed from the specimens preserved in 3%(v/v) glutaraldehyde, 10%(v/v) formalin or in TAF.

4 Discussion

DNA-based diagnostic methods have enabled the rapid and reliable identification of nematodes even from an individual sample disrupted with a micropipette tip (Harris et al. 1990; Cenis 1993; Powers and Harris 1993; Orui 1996) or a minute pin (Williamson et al. 1997). These methods, however, were somewhat difficult for unskilled researchers with minimal facilities because the nematode is prone to escape from the tip of a micropipette or minute pin in a drop of water. We have developed a simpler PCR-RFLP method than any preceding one by using a filter paper chip with the nematode remains as a DNA template. In the case where a nematode was disrupted by filter paper, the nematode could always be crushed. This method requires no technical skill and no time-consuming DNA-extracting

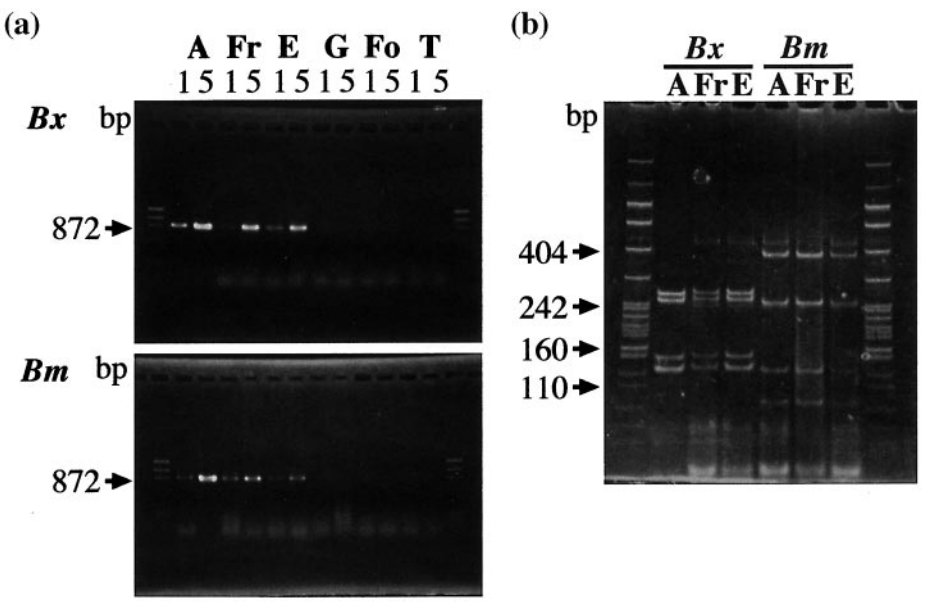

Fig. 4. (a) PCR-amplified products from 1-week-preserved specimen of one (1) or five (5) individuals of *Bursaphelenchus xylophilus* (*Bx*) and *B. mucronatus* (*Bm*) on 1.2% agarose gel. (b) *Hin*f I-digested PCR-RFLP patterns on 6% polyacrylamide gel. Samples are: A, alive nematode as control; Fr, specimens that are preserved at −20°C in distilled water; or at room temperature in E, 99.5% ethanol; G, 3% glutaraldehyde; Fo, 10% formalin; T, TAF. Molecular weight markers are shown in base pairs (bp)

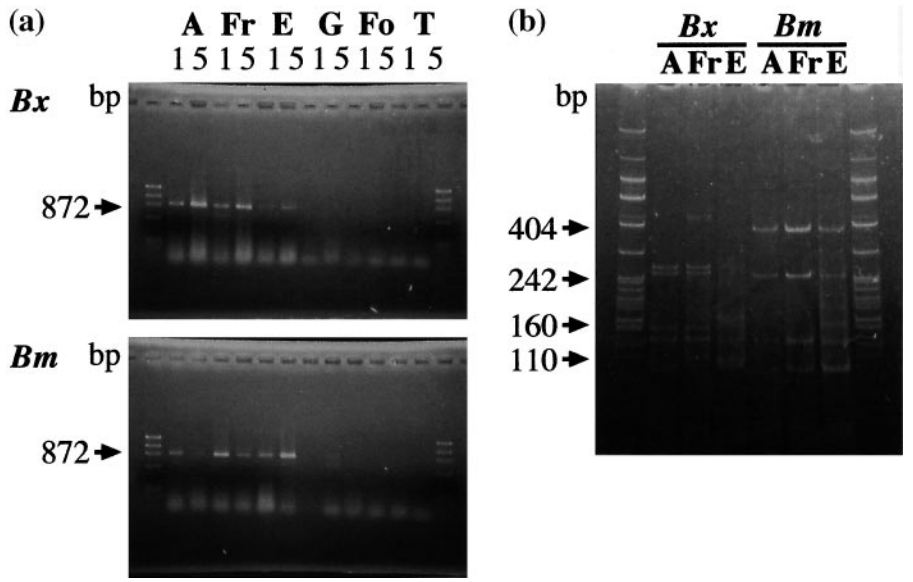

Fig. 5. (a) PCR-amplified products from 3-months-preserved specimens of one (1) or five (5) individuals of *Bursaphelenchus xylophilus* (*Bx*) and *B. mucronatus* (*Bm*) on 1.2% agarose gel. (b) *Hin*f I digested PCR-RFLP patterns on 6% polyacrylamide gel. Abbreviations are the same as Fig. 4. Molecular weight markers are shown in base pairs (bp)

steps, and the PCR products yielded were quite enough for subsequent RFLP or sequencing analysis. This method also proved to be applicable to field samples, and thereby showed practical value as a diagnostic skill that possibly could be applied to other nematode diseases.

There have been other reports concerning DNA-based detecting methods for pinewood nematodes. TARÉS et al. (1993, 1994) demonstrated that a satellite DNA probe is effective on a single nematode squashed on a nitrocellulose filter. Although this method was reported as rapid and efficient for PCR-based methods, examination by radioactive isotope and hybridization procedures are not always readily available, and are costly. In contrast, the present method requires only simple procedures, namely, an easy method of crushing nematodes, PCR, digestion with restriction enzyme, electrophoresis, and it is not costly.

About 90% or more of the trials with adult nematode individuals produced successful PCR products. When the second-stage juveniles were examined, however, the percentage of success decreased slightly to about 70–90%. When eggs served as the DNA template, PCR amplification brought about inconsistent results. This may be attributed to the difference in the number of cells or DNA copies in accordance with embryonic development. For example, about 70% of trials with eggs just before hatching resulted in success, whereas 50% or more trials with eggs just after being laid resulted in failure.

The methods of nematode preservation significantly affected the results of the PCR tests. When preserved in distilled water at −20°C, or in 99.5% ethanol at room temperature, the nematode specimens could be identified with the present PCR-RFLP method even after 3 months. However, the specimens that were preserved for only a week in 3% glutaraldehyde, in 10% formalin, or in TAF (all three of these solutions contain aldehyde) at room temperature produced no PCR products and were unsuitable for molecular biological identification. From the standpoint of DNA preservation, preserving nematodes in distilled water at −20°C or in 99.5% ethanol at room temperature was an excellent method. For

morphological observation, however, nematode specimens are usually fixed with, and then stored in aldehyde-containing solution which seems to denature the nematode DNA.

THOMAS et al. (1997) demonstrated that 5%-formalin-fixed nematodes could yield sufficient amounts of DNA template to allow PCR and even sequencing analysis, although the fixation period was limited to no longer than 2 days. DE GIORGI et al. (1994) found errors in newly polymerized DNA sequence of 2.5% formalin-preserved nematodes (preservation period was not shown) after PCR amplification. If the denaturation of nematode DNA could be recovered or nematode DNA could be protected by chemical treatment, integration of molecular and morphological analysis of the same specimen could be achieved. Not only preserved nematode specimens, but also the preserved specimens of various plants and animals which the nematodes inhabit, possibly could serve as the DNA template for PCR in the future.

Résumé

Une méthode simple de diagnostic du flétrissement des pins, basée sur la PCR-RFLP

En vue de diagnostiquer le flétrissement des pins, une méthode simple par PCR-RFLP a été mise au point pour identifier le nématode et pour le différencier de l'espèce voisine, à partir d'un seul échantillon vivant ou conservé. Les nématodes *Bursaphelenchus xylophilus* et *Bursaphelenchus mucronatus* ont été considérés. Un seul nématode dans 1 μl d'eau distillée a été déposé sur une lame de verre. Quand l'eau était presque complètement évaporée, le nématode était écrasé avec un fragment de papier filtre (1,5 mm × 1,5 mm) à l'aide d'une pince. Le papier était immédiatement placé dans le tampon de PCR. Les amorces utilisées étaient dirigées vers l'espaceur interne transcrit (ITS1, 5.8S, et ITS2) de l'ADN ribosomique. Le produit de la PCR était régulièrement obtenu à partir d'un seul nématode, et la digestion du produit avec *Hin*f I permettait de distinguer *B. xylophilus* et *B. mucronatus*. Cette méthode était simple et facile, pour diagnostiquer sans ambiguité le parasite. Elle était aussi applicable à des échantillons de nématodes conservés dans diverses conditions, sauf celles qui ont recours à des fixateurs à base d'aldéhyde.

Zusammenfassung

Eine einfache, PCR-RFLP gestützte Methode zur Diagnose der Kiefernwelke

Zur Diagnose der Kiefernwelke wurde eine einfache PCR-RFLP-Methode entwickelt, mit der zwei ähnliche Nematoden-Arten (*Bursaphelenchus xylophilus* und *Bursaphelenchus mucronatus*) auf der Basis von einzelnen lebenden oder konservierten Individuen unterschieden werden können. Ein einzelner Nematode wurde in 1 μl destilliertem Wasser aufgenommen und auf einen Objektträger gebracht. Wenn das Wasser weitgehend aufgetrocknet war, wurde der Nematode mit einem 1,5 mm × 1,5 mm grossen Filterpapierstück mit Hilfe einer Pinzette zerquetscht. Das Filterpapier mit den Nematodenüberresten wurde sofort als DNA-Template in den PCR-Puffer gegeben. Es wurden Primer verwendet, die die ITS1-Region, die 5,8 S-Region und die ITS 2-Region der ribosomalen DNS amplifizieren. Das PCR-Produkt wurde in der Regel von einem einzelnen Nematoden erhalten und die Verdauung des Produktes mit der Restriktionsendonuklease *Hin*f I erlaubte die Unterscheidung zwischen *B. xylophilus* und *B. mucronatus*. Diese Methode zur Diagnose des Erregers der Kiefernwelke ist einfach und zuverlässig. Sie konnte auch bei Nematoden angewendet werden, die unter verschiedenen Bedingungen konserviert waren, nicht jedoch bei Verwendung aldehydhaltiger Fixiermittel.

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