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Isolation of functional total RNA from *Tilia cordata* leaves and pollen

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Abstract: The conditions required for the isolation of high quality total RNA from European linden (*Tilia cordata*) leaves and pollen were determined. Pure total RNA was isolated from linden leaves utilizing a Qiagen plant mini kit, while the total RNA isolated from linden pollen using this method was degraded. Successful isolation of total RNA from both linden pollen and leaves, however, was achieved following TRIzol™ preparation of the total RNA. The total RNA isolated using TRIzol™ was contaminated with genomic DNA but treatment with the enzyme DNase, in solution or on-column, efficiently removed the genomic DNA. Furthermore, the conditions for the elimination of genomic DNA contamination on-column and isolation of pure total RNA from leaves were optimized. The isolated total RNA from both leaves and pollen was used successfully in first- and second-strand cDNA synthesis reactions and in a reverse transcription polymerase chain reaction (RT-PCR), demonstrating that the total RNA isolated using this method was functional. In conclusion, pure and functional total RNA from *T. cordata* leaves and pollen (27.8±7.9 μg g^{-1} leaves; 25.7±1.1 μg g^{-1} pollen) could be obtained and was suitable for application in further molecular biology studies.

Keywords: *Tilia cordata*; leaves; pollen; total RNA; RT-PCR; cDNA.

INTRODUCTION

The European species of linden, *Tilia cordata*, is the most common species of *Tilia*.[1] The outer layer of the linden pollen grain is composed of a complex polymer of carotenoids and carotenoid esters, which are quite resistant to chemical and biological decay. In addition to carotenoids, linden pollen also contains proteins, nucleic acids, carbohydrates and lipids, making it a complex biochemical

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source.[2] The European linden pollen is highly allergenic.[3,4] Seasonal exposure to allergens in linden pollen can cause asthma, allergic rhinitis and allergic conjunctivitis.[3–5] A study assessing the aeroallergen sensitization in an allergic population in Portugal found that linden tree is one of the most representative aeroallergens.[6] Contact dermatitis due to *T. cordata* has also been documented.[7,8]

Hitherto, very little biochemical research has been realized on the molecular properties of *T. cordata* leaves and pollen. Genetic analysis revealed that *T. cordata* has a disomic inheritance despite being an allopolyploid.[9] Regarding protein analysis of *T. cordata*, a putative 50 kDa allergen was isolated from linden pollen but has not yet been fully characterized.[5] An attempt was made to isolate RNA from *T. cordata* leaf and flower.[10]

To utilize molecular biology techniques in order to prepare a complementary DNA library from *T. cordata* genes, it is necessary to obtain a large quantity of pure and functional total RNA. In the present paper, a method for the total isolation of pure and functional RNA from *T. cordata* leaves and pollen is reported. Furthermore, the optimized conditions for the isolation of functional total RNA from both leaves and pollen of *T. cordata* are described.

EXPERIMENTAL

Chemicals. The RNeasy plant mini kit was from Qiagen (Hilden, Germany). TRIzol™ was from Invitrogen (Carlsbad, CA, USA). The enzymes DNase, DNA polymerase I and RNase H were from Qiagen, Stratagene (La Jolla, CA, USA), and Invitrogen, respectively. The RNA marker was from Sigma (St. Louis, MO, USA). The molecular biology reagents were supplied by Fermentas (Burlington, Canada), Invitrogen, or Qiagen. All other chemicals were from Sigma. *T. cordata* leaves were collected and stored at –80 °C, while the linden pollen was a generous gift from the Torlak Institute of Virology, Vaccines and Sera, Belgrade, Serbia.

Isolation of total RNA. For the total RNA isolation using the Qiagen RNeasy plant mini kit, up to 100 mg linden leaves or pollen were ground in liquid nitrogen, lysed, homogenized and applied onto spin columns according to the Qiagen protocol. The spin columns were eluted using 40 μl RNase-free water. For total RNA isolation using TRIzol™, from 300 mg up to 1 g linden leaves or pollen were used as the starting material and isolation was performed following the manufacturer's instructions. The obtained RNA pellet was resuspended in 150 or 200 μl RNase-free water per 300 mg leaves or pollen, respectively, and stored at –80 °C.

For DNase treatment in solution, 50 μl TRIzol™-prepared total RNAs were incubated with 2.5 μl of the stock solution (10 U μl^{-1}) of DNase for 15 min at room temperature followed by application onto a Qiagen spin column. For on-column DNase treatment, various amounts of isolated total RNA were applied onto Qiagen spin-columns and 10 μl of the stock solution of the DNase enzyme were added directly onto the column and incubated at room temperature for 15 min. Following on-column DNase treatment, the spin columns were washed and the total RNA eluted according to the Qiagen protocol.

The profile of total RNA was determined by agarose–formaldehyde denaturing electrophoresis.[10]

RNA purity, concentration and yield quantification. Concentration and purity of RNA were determined by measuring the absorbance at 260 nm (A_{260} nm) and the ratio between the

absorbance values at 260 nm and 280 nm (A_{260}/A_{280}), respectively. To determine the concentration of RNA, the following formula was used: $A_{260}\times 44$ μg $mL^{-1}\times$dilution factor.[11]

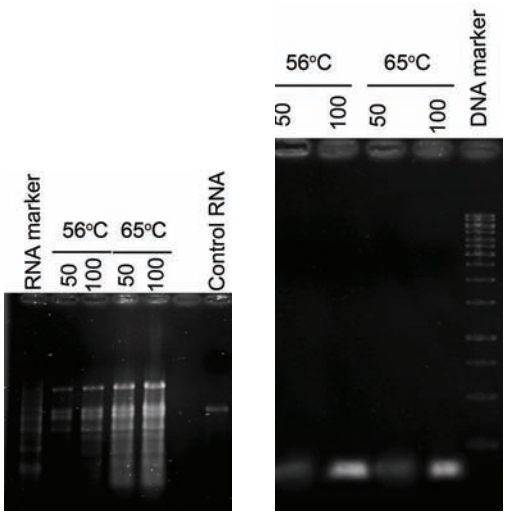
Total RNA isolated from linden leaves and pollen using TRIzol™ was used for first- and second-strand cDNA synthesis following the manufacturer's protocol (Stratagene).

Reverse transcription polymerase chain reaction (RT-PCR). Following first-strand cDNA synthesis reaction using total RNA isolated from leaves or pollen as the template, RNase H digestion was performed by incubating at 37 °C for 20 min. The PCRs were set up as follows: 5 μl RNase H-digested single stranded cDNA, 5 μl 10×PCR buffer, 4 μl 25 mM $MgCl_2$, 1 μl 10 mM dNTP mix, 1 μl (10 μM) of each primer (forward and reverse), 4U recombinant *Pfu* polymerase, and water up to 50 μl. After initial incubation at 95 °C for 2 min, 35 cycles of PCR amplification were performed with the following cycling parameters: 95 °C (30 s), 55 °C (1 min), 72 °C (2 min). A final extension at 72 °C for 5 min was performed. In order to amplify a fragment of the actin gene in *T. cordata*, the following actin primer sequences were used according to van den Berg *et al*.:[12] actin primer (forward): 5'-ACCGAAGCCCCT-CTTAACCC-3' and actin primer (reverse): 5′-GTATGGCTGACACCATCACC-3. Following RT-PCR, aliquots of the samples were migrated by 2 % agarose gel electrophoresis.

RESULTS AND DISCUSSION

Hitherto, only one article has been published describing an attempt to isolate total RNA from various plant sources, including the leaves and flower of *T. cordata*.[13] In that study, the authors did not specify the yield of total RNA obtained. Furthermore, an $A_{260/280}$ of 1.1 was reported for the total RNA isolated from leaves and flower, which is a value much lower than that expected for pure RNA (usually an $A_{260/280}$ in the range of 1.9 to 2.1), suggesting that the RNA isolated was impure.

In the present study, total RNA was initially isolated from the leaves of *T. cordata* using a Qiagen RNeasy plant mini kit. Agarose–formaldehyde gel electrophoresis showed distinct RNA bands, indicating that RNA molecules of various sizes had been isolated and that no RNA degradation had occurred (Fig. 1A). Total leaf RNA was isolated and eluted from the Qiagen spin-column using 50 mg of the starting material; however, a higher yield of total RNA was obtained using 100 mg of the starting material (Fig. 1A). Furthermore, an increased yield of total RNA was observed when the leaf lysates were incubated at 65 °C instead of 56 °C (Fig. 1A). As recommended in the protocol for Qiagen RNeasy plant mini kit, re-elution of the spin-column increased slightly the yield of isolated total RNA (data not shown). On the other hand, agarose–formaldehyde gel electrophoresis for the total RNA isolated from *T. cordata* pollen indicated RNA degradation (Fig. 1B). Even under conditions which seemed to improve the yield of total RNA isolation from leaves, using a higher amount of starting material (100 mg) or increasing the temperature during lysis of the sample (65 °C), the total RNA isolated from pollen was completely degraded (Fig. 1B).



(A) (B)

Fig. 1. Electrophoretic profile after isolation of total RNA from linden leaves and pollen using Qiagen plant mini kit. Total RNA from 50 or 100 mg of *T. cordata* leaves or pollen was purified with a Qiagen plant mini kit. The RNA samples were analyzed by 1.2 % formaldehyde–agarose electrophoresis and ethidium bromide staining. RNA marker, RNA control (1.6 kb), and/or DNA marker were run as size standards. *T. cordata* leaves (A) or pollen (B) were ground in liquid nitrogen and lysates were incubated at 56 or 65 °C for 5 min prior to homogenization and application onto Qiagen spin columns.

Subsequently, total RNA isolation using the commercial reagent TRIzol™ followed by chloroform extraction and 2-propanol precipitation was attempted. Using this method, total RNA was isolated from both linden leaves and pollen (Fig. 2) However, the total RNA obtained using this method was contaminated with genomic DNA. In order to eliminate the genomic DNA, DNase treatment was performed in solution or on-column, and total RNA was eluted after Qiagen RNA column clean-up. DNase treatment, both in solution and on-column, was effective in removing the genomic DNA, whereby the total RNA isolated from either leaves or pollen remained intact (Fig. 2).

The yields of total RNA isolated from leaves and pollen after DNase treatment to remove the genomic DNA contamination were 27.7 μg g^{-1} for leaves and 25.7 μg g^{-1} for pollen. The isolated RNA was of adequate purity (average A_{260}/A_{280} of 1.91 for leaves and 1.94 for pollen). Before DNase treatment, the total RNA isolated from both leaves and pollen was impure with average A_{260}/A_{280} values of 1.58 and 1.11, respectively (Table I).

While the yields of isolated total RNA from *T. cordata* leaves and pollen were similar, the relatively low yields of isolated total RNA compared to the total RNA isolated from other plant sources can be explained by the fact that linden

represents a difficult source due to its chemical and molecular composition. Linden pollen is an especially difficult starting material because it contains a variety of molecules, including carotenoids, flavonoids, phenols and lipids, all of which can make the RNA isolation process difficult. Indeed, the isolation of the RNA from *T. cordata* pollen with the Qiagen plant mini kit, unlike from *T. cordata* leaves, was unsuccessful, rendering almost completely degraded RNA (Fig. 1B). Furthermore, even using TRIzol™, which allowed for the successful isolation of total RNA from both leaves and pollen, some RNA isolated from pollen was still degraded. Hence, *T. cordata* leaves and pollen probably differ in their molecular composition and may require different strategies for the most effective biochemical manipulation and possible improvement in the yield of isolated total RNA.

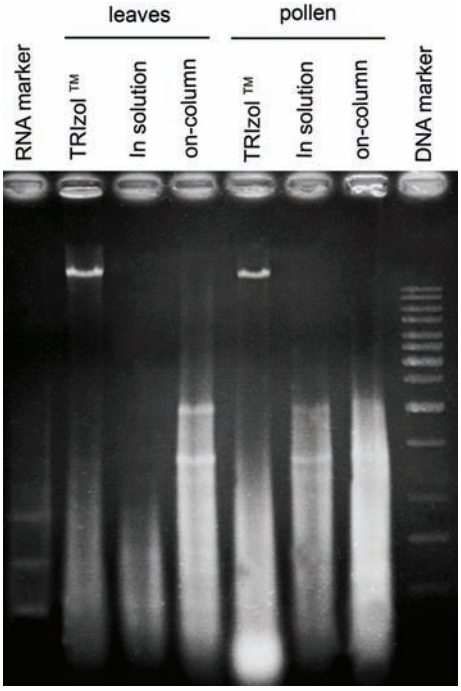
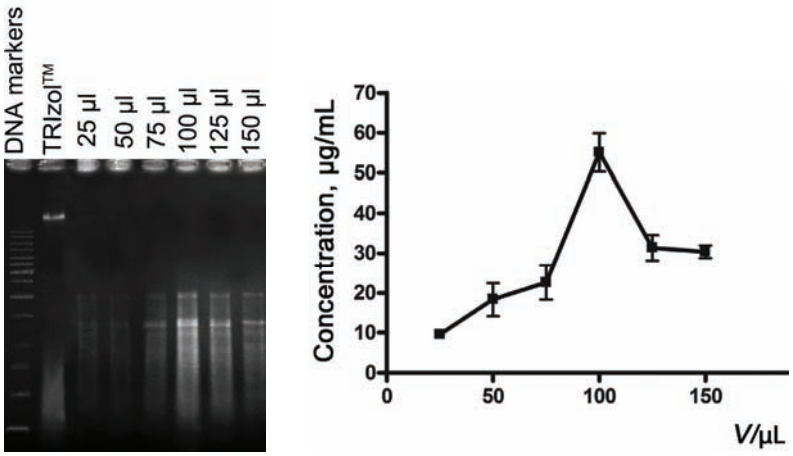


Fig. 2. Electrophoretic profile after isolation of total RNA from linden leaves and pollen using TRIzol™. *T. cordata* leaves or pollen (300 mg) were ground in liquid nitrogen and the total RNA was isolated using TRIzol™ and chloroform extraction. DNase treatment was performed either in solution or on-column. The RNA samples eluted after Qiagen on-column clean-up were analyzed by 1.2 % formaldehyde–agarose electrophoresis and ethidium bromide staining. RNA and DNA markers were run as size standards.

TABLE I. Concentration and purity of the total RNA isolated from *T. cordata* leaves and pollen with TRIzol™, before and after treatment with DNase (in solution or on-column)

Specimen	Sample	A_{260}/A_{280}
Leaves	TRIzol™	1.58±0.19
	After DNase treatment in solution	1.74±0.09
	After DNase treatment on-column	1.91±0.05
Pollen	TRIzol™	1.11±0.16
	After DNase treatment in solution	1.71±0.29
	After DNase treatment on-column	1.94±0.21

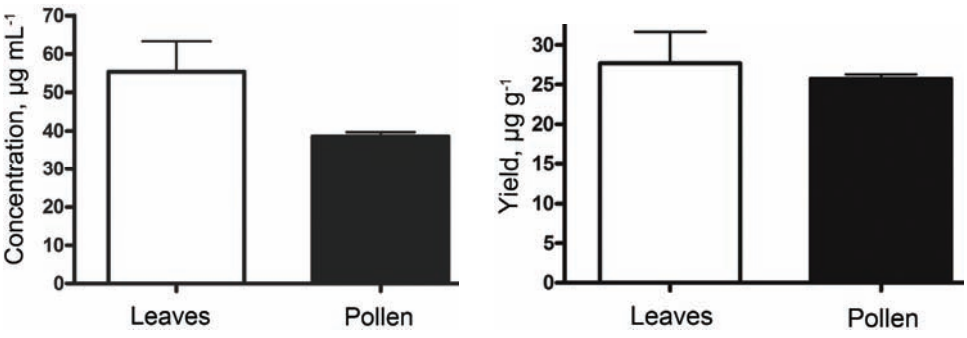
The next goal was to optimize the isolation of total RNA and elimination of genomic DNA using on-column DNase treatment. Up to 100 μg RNA can be loaded onto Qiagen plant mini kit columns and hence the optimal amount of TRIzol™-prepared total RNA that could be loaded onto a Qiagen spin column in order to efficiently remove the genomic DNA and isolate the highest possible amount of pure total RNA was determined. Various amounts of TRIzol™-prepared total RNA isolated from *T. cordata* leaves, ranging from 25 up to 150 μl, were applied onto Qiagen spin columns, treated with DNase enzyme for 15 min at room temperature, and eluted with RNase-free water. Samples from each on--column treatment were loaded and migrated on 1 % agarose gel. Genomic DNA was efficiently removed and pure RNA that was not degraded was obtained with samples, ranging from 25 to 150 μl, of TRIzol™-prepared total RNA applied for on-column DNase treatment (Fig. 3A). However, the optimal amount of TRIZOL™-prepared total RNA applied for on-column DNase treatment that resulted in the highest yield of eluted RNA was 100 μl (Fig. 3A). Furthermore, using three independent measurements of total RNA concentration by spectrophotometry (A_{260}), it was confirmed that applying 100 μl of TRIzol™-prepared RNA for on-column treatment was an optimal amount (Fig. 3B). Interestingly, for amounts over 100 μl of TRIzol™-isolated total RNA applied on-column, there was a decrease in the yield of isolated pure RNA.



(A) (B)

Fig. 3. Electrophoretic profile after optimization of the total RNA isolation following on--column DNase treatment. DNase treatment was performed on Qiagen mini spin columns after applying 25, 50, 75, 100, 125, or 150 μl of the total isolated RNA. A) Total RNA samples were analyzed by 1 % agarose electrophoresis and ethidium bromide staining. DNA markers were run as size standards. B) Yield of total RNA eluted (μg mL^{-1} eluted volume) after on-column treatment with DNase, determined by measuring the concentration of eluted RNA using spectrophotometry (A_{260}).

Concentration and purity of total RNA isolated from leaves and pollen using TRIzol™, before and after DNase treatment, on-column or in solution, were determined by measuring in triplicate the absorbance at 260 nm (A_{260}) and the ratio between absorbance at 260 and 280 nm (A_{260}/A_{280}), respectively. However, following DNase treatment on-column, the isolated RNA was pure with an average A_{260}/A_{280} of 1.91 for leaves and 1.94 for pollen. In terms of concentration, the A_{260} values of the TRIzol™ samples prior to DNase treatment were not taken into account because the contamination with genomic DNA obviously leads to overestimation of the real RNA concentration. Three measurements of the A_{260} value following on-column DNase treatment were used to calculate the average concentration of eluted total RNA following on-column DNase treatment, which was approximately 55.4±15.8 μg mL^{-1} for the total RNA isolated from leaves and 38.6±2.2 μg mL^{-1} for the total RNA isolated from pollen (Fig. 4A). These concentration values were further used to determine the average yield of isolated total RNA and a yield of 27.7±7.9 μg g^{-1} starting material was obtained for leaves compared to 25.7±1.1 μg g^{-1} starting material for pollen (Fig 4B).

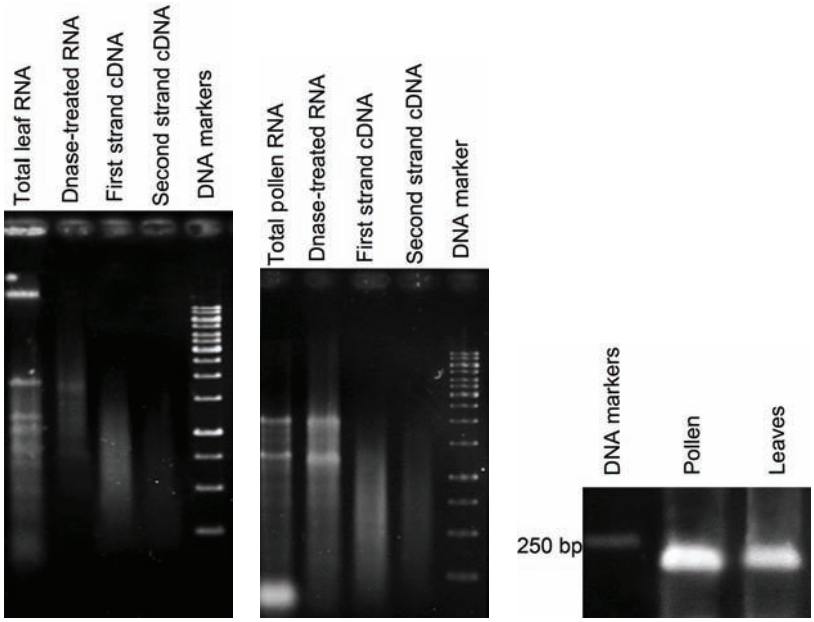


(A) (B)

Fig. 4. Concentration, purity, and yield of total RNA isolated from *T. cordata* leaves and pollen. A) Concentration of total RNA (μg mL^{-1}) isolated from *T. cordata* leaves or pollen using TRIzol™ and digested on-column with DNase enzyme. B) Yield of total RNA (μg g^{-1} of starting material) isolated from *T. cordata* leaves or pollen using TRIzol™ and digested on-column with DNase enzyme. Values were obtained using RNA samples from different isolations and three independent measurements.

To check the functionality of the isolated RNA, cDNA synthesis was performed using the total RNA isolated from linden leaves or pollen as a template. Using total RNA isolated from leaves with TRIzol™ and digested on-column with DNase, first- and second-strand cDNA synthesis reactions were performed, which resulted in the synthesis of single- and double-stranded cDNA, respectively, as shown by the broad range smears on the agarose gel after electrophoresis (Fig. 5A). Similar results were observed using total RNA isolated from linden pollen (Fig. 5B). To confirm further the functionality of the RNA preparations,

isolated total RNA was used in an RT-PCR reaction aimed at detecting and amplifying a fragment of the actin gene in *T. cordata* leaves and pollen (Fig. 5C). Indeed, a PCR-amplified fragment of identical size of less than 250 bp was observed in the RT-PCR using either total RNA isolated from leaves or pollen as template, showing that the isolated total RNA was functional.



(A) (B) (C)

Fig. 5. Electrophoretic profile during cDNA synthesis and RT-PCR using total RNA isolated from *T. cordata* leaves and pollen. A) Total RNA isolated from *T. cordata* leaves using TRIzol™ was digested on-column with DNase and used as the template for first- and second-strand cDNA synthesis. B) Total RNA isolated from *T. cordata* pollen using TRIzol™ was digested on-column with DNase and used as the template for first- and second-strand cDNA synthesis. C) RT-PCR to amplify a fragment of the actin gene was realized using total RNA isolated from leaves or pollen. The samples were analyzed by 1 % agarose (A and B) or 2 % agarose gel electrophoresis (C) and ethidium bromide staining. DNA markers were run as size standards.

CONCLUSION

In the present paper, conditions for the isolation of functional RNA from *T. cordata* leaves and pollen are reported. This is the first report of the isolation of total RNA from *T. cordata* pollen. The total RNA isolated from leaves and pollen using TRIzol™ and DNase on-column digestion was functional, as confirmed by cDNA synthesis and RT-PCR reactions of the linden leaves and pollen actin gene (Fig. 5C). Stemming from these results, it was demonstrated that it is possible to isolate RNA from *T. cordata* leaves and pollen and, furthermore, that the ob-

tained total RNA could be of use for further molecular biology research aimed at characterizing the biochemical properties of *T. cordata*.

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И З В О Д

ИЗОЛОВАЊЕ ФУНКЦИОНАЛНЕ УКУПНЕ РНК ИЗ ЛИШЋА И ПОЛЕНА ЛИПЕ (*Tilia cordata*)

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Успостављени су услови за изоловање укупне РНК из лишћа и полена европске липе (*Tilia cordata*). Коришћењем комерцијално доступног прибора за изоловање РНК из биљака изолована је чиста укупна РНК из лишћа липе, док је коришћењем исте методе добијена деградирана РНК из полена липе. Успешно изоловање РНК из лишћа и полена је добијено коришћењем ТРИзол реагенса. РНК изолована овим методом је контаминирана геномском ДНК, која је успешно елиминисана коришћењем ензима ДНазе. Даље су оптимизовани и услови уклањања геномске ДНК помоћу ДНазе. Изолована укупна РНК из оба извора је даље успешно искоришћена за синтезу првог и другог ланца клонске ДНК, као и у реверзно-транскриптивној PCR реакцији, доказујући тиме да је коришћењем овог метода изолована функционална укупна РНК. У закључку, добијена је чиста и функционална РНК из лишћа и полена *T. cordata* (27,8±7,9 μg g^{-1} лишћа; 25,7±1,1 μg g^{-1} полена) која се може користити у даљим молекуларно-биолошким истраживањима.

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