The use of herbarium specimens in DNA phylogenetics: Evaluation and improvement

Savolainen, Vincent; Cuenoud, Philippe; Spichiger, Rodolphe; Martinez, Maria D. P.; Crèvecoeur, Michèle; Manen, Jean-François

Pl. Syst. Evol. 197: 87–98 (1995)

Plant Systematics and Evolution

The use of herbarium specimens in DNA phylogenetics: evaluation and improvement

VINCENT SAVOLAINEN, PHILIPPE CUÉNOUD, RODOLPHE SPICHIGER, MARIA D. P. MARTINEZ, MICHÈLE CRÈVECOEUR, and JEAN-FRANÇOIS MANEN

Received September 26, 1994; in revised version February 13, 1995

Key words: Herbarium specimens, molecular phylogenetics, ancient DNA, PCR techniques.

Abstract: During the last few years we have been confronted with the need to use herbarium specimens in the molecular phylogeny studies, since it is generally difficult to obtain living material of some rare species. Ancient DNA has been sequenced, and there are also reports on successful DNA amplification from herbarium specimens. However, it is not easy to obtain amplified DNA from the first herbarium sample tested. In this paper, experiments are described about trials of DNA amplification from two to 151-year-old herbarium specimens of plant species we needed for our projects. Of the 17 herbarium samples tested only two allowed DNA amplification under standard DNA isolation conditions. Different types of PCR inhibiting activities were demonstrated in DNA extracts. In some of the extracts there was extremely low concentration of template with satisfactory quality. In some instances, PCR inhibiting activities were successfully removed by treating them either with insoluble polyvinylpyrrolidone or by adding bovine serum albumin (BSA) to the amplification mixture. However, some PCR-inhibiting activities were resistant to the treatments described above. When the concentration of template was very low, a second PCR amplification with internal primers was necessary to increase the amount of DNA for sequencing. Nevertheless, contamination of either DNA extract or amplification mixture were sometimes observed, and consequently precautions were taken to minimize them. Finally, successful amplification was obtained in eight samples out of the 17 examined.

In the past decade, phylogenetic studies based on DNA comparison has emerged as a new field of biology, partly as a result of the development of the polymerase chain reaction (PCR) and DNA sequencing methods which allow the rapid accumulation of large data sets. All kinds of organisms are studied through DNA evolution, and global projects are undertaken, such as those for plants (CHASE & al. 1993). However, an important difficulty is the collection of living plant samples, most interesting taxa from the point of view of evolution are often rare and geographically restricted. Thus, herbarium specimens potentially represent an invaluable source of material for molecular analysis, encompassing all collected taxa. Moreover, research in ancient DNA has recently generated much excitement

(Lewin 1994) as a result of the reported successes in DNA amplification and sequencing from plant and animal samples several thousands or millions of years old (Höss & Pääbo 1993, Cooper & al. 1992, Cano & al. 1993, Hagelberg & Clegg 1993, Janczewski & al. 1992, DeSalle & al. 1992, Golenberg & al. 1990, Soltis & al. 1992). Such data may indicate that DNA extraction from one-hundred-year-old herbarium specimens may not be too problematic. However, few papers report such experiments (e.g., Doyle & Dickinson 1987, Pyle & Adams 1989, Cano & Poinar 1993, Taylor & Swann 1994, Adams & al. 1994, Loockermann & Jansen 1995). While investigating the molecular phylogenetics of *Celastrales* (Spichiger & al. 1993, Savolainen & al. 1994) and the evolution of the genus *Ilex*, we decide to follow more rigorous methods for extracting DNA of herbarium specimens. We examined 17 herbarium specimens, collected from two to 151 years ago and representing a variety of different conditions of preservation (Table 1). In order to preserve the herbarium collections, only specimens having enough leaves have been sampled. Since the quality and quantity of DNA, as well as the presence of PCR inhibiting activities are the main problems in extracting ancient DNA (Pääbo 1989, 1991; Pääbo & al. 1989; Golenberg 1991), four methods of DNA extraction were used. PCR cross-reactions were performed to investigate inhibiting activities in the herbarium DNA extracts. For removing the inhibitors and for increasing the PCR efficiency, different protocols of DNA amplification were followed. Transmission electron microscopy of chloroplasts was performed on *Ilex* herbarium specimens collected 10 and 68 years ago, followed by DNA extraction and amplification to check for the putative conservation of DNA in chloroplasts.

Material and methods

Herbarium specimens. The 17 herbarium specimens used in these experiments are listed in Table 1.

DNA extraction. In order to avoid contamination of PCR products, DNA extraction and mix preparation were carried out in a separate laboratory, used only for this purpose. An extraction control (extraction processed without a plant sample, checked by a subsequent PCR run) was always carried out. For this study four different DNA extraction procedures were used:

1. The current CTAB (Hexadecyl-trimethyl-ammonium-bromide) extraction method was carried out using a modified protocol of Webb & Knapp (1990). Approximately 50 mg of liquid nitrogen ground tissue was rapidly mixed in an Eppendorf tube containing 700 µl of hot extraction buffer (2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 25 mM EDTA, 0.2% mercaptoethanol, pH 8), and incubated at 60 °C for 30 min. At the end of the extraction, the DNA pellet was suspended in 20 µl of TE8 (10 mM Tris-HCl, 1 mM EDTA, pH 8).

2. The DTAB (Dodecytrimethyl-ammonium-bromide) method of Gustincich & al. (1991) was modified as follows: 50 mg of liquid nitrogen ground tissue was rapidly mixed in an Eppendorf tube containing 700 µl of hot extraction buffer (5.5% DTAB, 1 M NaCl, 70 mM Tris-Hcl, 30 mM EDTA, pH 8) and incubated at 60 °C for 30 min. After chloroform extraction, 1.7 volumes of 0.5% CTAB, 40 mM NaCl was added and mixed at room temperature, leading to DNA precipitation within 5 min. The original protocol was then followed and at the end of the DNA extraction, the pellet was suspended in 20 µl of TE8.

3. A method based on guanidine thiocyanate and silica (see also Carter & Milton 1993, Höss & Pääbo 1993) was used. Approximately 25 mg of liquid nitrogen ground tissue was rapidly mixed in an Eppendorf tube containing 500 µl of hot extraction buffer (4 M guanidine thiocyanate, 50 mM Tris-HCl, 20 mM EDTA, pH 7.5) and incubated at

Table 1. List of herbarium specimens used. [a] Mnemonic three-letter acronyms according to WEBER (1982)

No.	Species and family[a]	Age (year)	Voucher
1	*Cardiopteris lobata* WALL. (CRP)	109	ZOLLINGER 446 G
2	*Alzatea verticillata* GRAHAM (CEL)	91	ULE 6750 G
3	*Sphenostemon pauciflorum* VAN STEENIS & ERDTM. (AQF)	58	CLEMENS 9828 G
4	*Lophopyxis* spec. HOOK (LPX)	64	KAJEWSKI 2269 G
5	*Pentaphylax euryoides* GARDN. & CHAMP. (PHC)	56	TAAM 917 G
6	*Microdesmis puberula* HOOK (PDA)	4	CARVALLIO 4250 G
7	*Stackhousia dielsii* PAMPAN. (STK)	12	STRID 20824 G
8	*Staphylea colchica* STEV. (STP)	27	Ign. 380792 G
9	*Celastrus hindsii* BENTH. (CEL)	56	TAAM 759 G
10	*Cyrilla racemiflora* L. (CYR)	151	Ign. 399609 G
11	*Ilex aquifolium* L. (AQF)	29	WODLAND 536 G
12	*Parnassia palustris* L. (SAX)	92	Ign 9.1902 G
13	*Ilex aquifolium* L. (AQF)	68	SCHUMAKER G
14	*Ilex pachyphylla* MERRILL (AQF)	44	SULIT 12544 G
15	*Acacia* spec. nova (FAB)	2	FORTUNATO & al. 3677 G
16	*Forsteronia pubescens* A. D. C. (APO)	2	RAMELLA & al. 3058 G
17	*Ilex aquifolium* L. (AQF)	10	VOGT 2376 G

60 °C for 30 min. After phenol and chloroform extractions, 10 µl of a silica particles suspension (Prep-A-gene, Biorad) was added and the supplier's protocol followed. The DNA was finally eluted in 20 µl of TE8.

4. The DNA extraction Protocol of DOYLE & DOYLE (1990) as modified by TABERLET & al. (1991) was used. Approximately 20 mg of liquid nitrogen ground tissue was rapidly mixed in an Eppendorf tube containing 200 µl of hot CTAB buffer (2% CTAB, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl pH 8) and incubated at 60 °C for 60 min. After phenol and chloroform extractions, the buffer was filtered in Microcon 30 tubes (Amicon, Berkeley) and washed twice with 400 µl water. The DNA was then eluted from the membrane with 50 µl water.

PCR amplification. Figure 1 shows the amplified chloroplast DNA (cpDNA) fragments and the primers used in this survey. The primer pair 2 and 5 amplifies the *atp*B-*rbc*L spacer of approximately 900 bp. The primer pair 16 and 12 amplifies a 369 bp fragment of the *rbc*L coding sequence. The PCR reaction includes 1 µl of DNA extract in a 25 µl standard PCR mix (50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.1% gelatin, 2 mM $MgCl_2$, 200 µM of each dNTP, 0.25 µM of the primers and 0.5 units of Perkin Elmer Taq DNA-polymerase). Each sample was cycled through 35 thermocycles each consisting of a denaturation step of 1 min at 93 °C, an annealing step of 30 sec at 50 °C, and an extension step of 1 min at 72 °C. A final extension step of 5 min at 72 °C was performed. PCR products were separated in a 1.5% agarose gel stained with ethidium bromide, and observed under UV light. Two PCR controls were always included: a positive control consisting of a diluted DNA extract from fresh leaves of *Ilex aquifolium* and a negative control consisting of PCR without template DNA. These reaction conditions were subsequently modified as stated below. In order to increase amplification efficiency double-PCR were run: 1 µl of the DNA extract was amplified using the primer pair 30 and 31, then 1 µl of the PCR product was used as template for a second PCR run, using the internal primer pair 2 and 5 (Fig. 1). To avoid contamination each PCR product (except the Taq polymerase and the

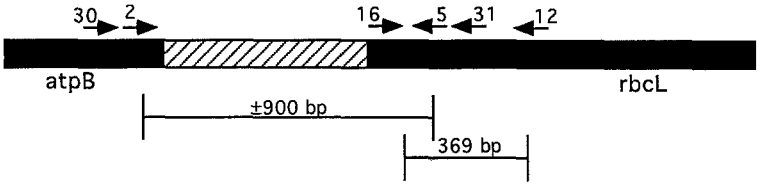

Fig. 1. Map of the cpDNA amplified region (the *atp*B-*rbc*L spacer and the beginning of the *rbc*L coding sequence) with the position of the primers used (represented by arrows). The sequences of primers are: N° 2. 5'GAAGTAGTAGGATTGATTCTC 3'; N° 5. 5'TACAGTTGTCCATGTACCAG 3'; N° 12. 5'TCAACTTGGATACCGTGAGG 3'; N° 16. 5' TATCTTGGCAGCATTCCGAG 3'; N° 30. 5' GCATCGTCCTTTGTAACGATC 3'; N° 31. 5'TTTCAAGCGTGGAAACCCCAG 3'

DNA extracts) and reagent was treated by exposure under UV light for 10 min (SARKAR & SOMMER 1990).

Gross-PCR reactions. In order to detect PCR inhibiting activities in DNA extracts, PCR cross-reactions were carried out using 1 µl of each herbarium DNA extract with 1 µl of a diluted positive DNA extract from fresh leaves of *Ilex aquifolium* as template. If the expected DNA fragment was not amplified, it means that the herbarium extract contains inhibitors of the PCR reaction and they interfere with the amplification reaction.

Removal of potential inhibiting activities. When PCR inhibiting activities were detected in herbarium DNA extracts, they were treated with 1 µl of a Polyclar AT suspension (polyvinylpyrrolidone insoluble polymer, Serva, 100 mg/ml) in order to remove phenolic compounds (LOOMIS & BATAILLE 1966). With the same purpose, PCR reaction conditions were modified either by increasing the $MgCl_2$ concentration from 2 mM to 6 µM, by adding BSA at 0.004% (final concentration) or by diluting the herbarium DNA extract (1/100).

Electron transmission microscope. Small pieces of fresh leaves, leaves dried in silica gel, and from herbarium specimens collected 10 and 68 years ago, of *Ilex aquifolium* were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 24 h. After three 10 min washings in cacodylate, the samples were postfixed for 1 h in 1% osmium tetroxide, washed again, dehydrated through a graded ethanol series and embedded in Spurr's resin. Ultrathin sections (60 nm thick) were cut and stained with 5% aqueous uranyl acetate in water for 10 min followed by lead citrate (REYNOLDS 1963) for 12 min. They were examined with a Zeiss EM10 electron microscope. The samples from the two *Ilex aquifolium* sheets collected 10 and 68 years ago, were taken from the same leaves as those used for the DNA extraction.

Results

Comparison of DNA extraction methods. In approximately 10% of the samples, DNA from herbarium specimens was extracted and successfully amplified using the CTAB or the DTAB extraction method. However, large amounts of blackish material always co-precipitated with DNA at the precipitation step. It was generally impossible to remove this oxidized material, which can influence PCR amplification. Therefore DNA extractions were tested that avoid the precipitation step. Samples were extracted by using guanidine-silica method or Microcon method, and then were submitted to PCR amplification of the *atp*B-*rbc*L spacer (± 900 bp) (primers 2 and 5, Fig. 1). The two extraction procedures gave the same results, no PCR products were detected, except for *Microdesmis* (sample 6) and *Ilex* (sample 17). This experiment and the subsequent ones are summarized on Table 2.

Table 2. DNA amplification from herbarium specimens with different PCR reaction conditions. [a] Samples as in Table 1. Different fragments were amplified by PCR, using either primers 2 and 5 (± 900 bp, BSA, 1/100, 14×, Double-PCR), or primers 16 and 12 (369 bp; see Fig. 1). The samples were treated with Polyclar AT, then BSA (0.004% final concentration) was added to the 25 µl of PCR reaction (BSA), or the DNA extracts were diluted 1/100 (1/100) or concentrated 14 times (14×). Finally, the PCR product, amplified with primers 30 and 31, were used as template for a subsequent amplification with internal primers 2 and 5 (double-PCR). The presence of a PCR product visible in a 1.5% agarose gel stained with ethidium bromide is indicated by +, an undetectable PCR product is indicated by –, and the non-tested PCR reaction conditions are indicated by nt

Sample[a]	± 900 bp	BSA	1/100	14×	Double-PCR	369 bp
1	–	–	–	–	–	+
2	–	–	–	–	–	–
3	–	–	–	–	–	–
4	–	–	–	–	+	+
5	–	–	–	–	–	–
6	+	nt	nt	nt	+	+
7	–	–	–	–	+	–
8	–	–	–	–	–	–
9	–	–	–	–	–	–
10	–	–	–	–	–	–
11	–	–	–	–	–	+
12	–	–	–	–	–	–
13	–	–	–	–	–	–
14	–	–	–	–	–	–
15	–	–	nt	nt	+	nt
16	–	–	nt	nt	+	nt
17	+	nt	nt	nt	nt	nt

Testing for PCR-inhibiting activities in herbarium extracts. In order to test inhibiting activities, the samples (1 to 14, Table 1) were tested with a PCR cross-reaction using a positive DNA amplification of DNA extract from fresh *Ilex* leaves. Many of the samples inhibited the amplification of DNA extracted from fresh leaves of *Ilex aquifolium*, except for samples 1, 4,–7, 11, and 13. Since these samples dit not seem to show inhibiting activities, they were submitted to PCR at higher concentration (14 times). The amplifications were still unsuccessful, except for samples 6 and 17. This result might indicate that these extracts contain none or very few templates (see below).

Removal of PCR-inhibiting activities. Those samples which showed inhibiting activities (1, 3, 8–10, 12, and 14) were treated with Polyclar AT, followed by PCR cross-reaction with DNA extract from fresh *Ilex aquifolium*. Modifications of the PCR cross-reaction conditions were also tested by adding BSA (0.004%, final concentration), by increasing the magnesium concentration to 6 mM, and by diluting 1/100 the DNA template. The results of these treatments are summarized in Fig. 2. Removal of PCR-inhibition was successful in some cases (samples 3, 8, 9, 12, and 14), but not in all. The addition of BSA was particularly effective. The analysis of the PCR inhibitor-clearing experiments (Fig. 2) shows that there is evidence for different kinds of inhibitors. From the inhibiting extracts (samples 2, 3,

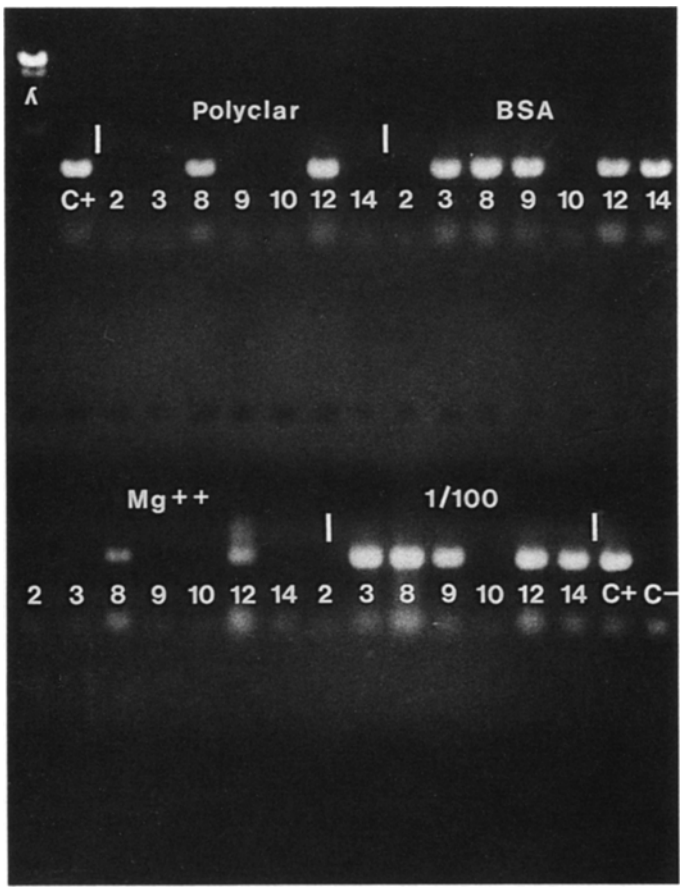

Fig. 2. Detection and removal of PCR-inhibiting activities in DNA extracts. Cross-PCR reactions: a 25µl PCR reaction was carried out using 1 µl of herbarium DNA extract (samples 2, 3, 8–10, 12, 14) mixed with 1 µl of a positive DNA extracted from fresh leaves of *Ilex aquifolium* as template, in four different assays: (1) the herbarium extract was treated with a Polyclar AT suspension (Polyclar); (2) BSA was added to the amplification mix (BSA); (3) magnesium concentration was increased to 6 mM (Mg^{2+}); (4) the herbarium DNA extract was diluted 1/100 (1/100). The products of PCR were run in a 1.5% agarose gel stained with ethidium bromide (C+ is the positive control, with DNA from fresh leaves of *Ilex*, and C– is the negative control, without any DNA template). When DNA fragment was not amplified, this means that the inhibitor activities present in the herbarium extracts had not be removed

8,–10, 12, and 14) Polyclar AT removed the inhibition from two of the samples examined (8 and 12). To a lesser extent 6 mM magnesium had the same effect. BSA removed the inhibition of other three samples, the same was observed also by diluting 1/100 the extract. In the two remaining samples (2 and 10) none of these treatments were able to remove inhibition. Thus we can suggest that at least three classes of inhibitors with different properties are present in the herbarium material examined.

PCR of herbarium specimens after removal of PCR inhibitors. The non-diluted or 1/100-diluted herbarium extracts (1 to 14) treated with Polyclar AT were amplified with primers 2 and 5, in a PCR reaction mixture containing BSA.

However, PCR amplification was unsuccessful. Since PCR inhibitors had been previously removed in several samples, an explanation for the failure of amplification could be that our DNA extracts contain very few or no DNA templates. With primer pair 2 and 5, the maximum amplification ratio obtained was between 10^7 and 10^8 which is equivalent, for 35 cycles, to an average efficiency of 70 to 80% per cycle. The increase in cycles did not result in an increase of PCR products. In agarose gels stained with ethidium bromide, a visible 900 bp DNA band (approximately 10 ng) contains at least 10^{10} DNA molecules. Thus it must be at least 100-1000 template molecules at the beginning of the reaction, in order to be able to observe a DNA band in the gel after 35 PCR cycles. This value is probably underestimated in routine experiments. If there is less than 100 template molecule no band would be visible. When there was a lower amount of DNA template, double PCR runs were carried out, firstly by using primers 30 and 31, and secondly by using the internal primers 2 and 5 (Fig. 1). Following the second PCR run, the amplification was successful for *Lophopyxis* (sample 4), *Microdesmis* (sample 6), *Stackhousia* (sample 7), *Acacia* (sample 15), and *Forsteronia* (sample 16). It is important to remember here that these amplifications were performed with materials decontaminated through exposure to UV light (see under Material and methods). Otherwise, we obtained amplification of contaminants as revealed by further sequencing (see below under Discussion). The failure of amplify a 900 bp fragment may be explained by the fact that the average size of the DNA template molecules in the recalcitrant herbarium specimens is shorter than 900 bp. Therefore amplification of a shorter cpDNA fragment by a simple PCR was undertaken (Table 1, Fig. 1). Primer pair 16 and 12 successfully amplified a 369 bp fragment of the *rbc*L coding sequence in *Lophopyxis* (sample 4) and *Microdesmis* (sample 6), as well as in two other samples such as *Cardiopteris* (sample 1) and *Ilex aquifolium* (sample 11), in which the amplification of the 900 bp fragment had previously failed.

Cytology of chloroplasts in herbarium samples. Figure 3 shows some electron micrographs of chloroplasts from leaf sections of *Ilex aquifolium*. The chloroplasts from fresh leaves contain many grana and osmiophilic plastoglobuli (Fig. 3 A, C). In samples dried in silica gel the chloroplasts are distorted but their thylakoids and envelopes are well preserved (Fig. 3 B, D). However, in contrast with freshly fixed chloroplasts, the membranes and the plastoglobuli of silica gel-dried samples are not electron dense, but appeared to be electron transparent (Fig. 3 C, D). We have no explanation for this phenomenon, but the rapid drying process of the silica gel method modifies the lipid bilayer phase of thylakoid membranes and the lipids of plastoglobuli, thus preventing access of osmium tetroxide to the membranes. The herbarium samples which were probably not so rapidly dried do not exhibit this peculiarity at their membrane level. High quality DNA was readily extracted from the fresh and the silica gel dried samples. In the herbarium specimens, DNA was obtained and amplified from the 10-year-old sample but not from the 68-year-old sample. The 10-year-old sample contained well preserved chloroplasts membranes (Fig. 3 E), whereas chloroplasts from the 68-year-old sample was not so well preserved and their membranes appeared diffuse (Fig. 3 F). It is important to note that the appearance of these chloroplasts might also result from other reasons than old age.

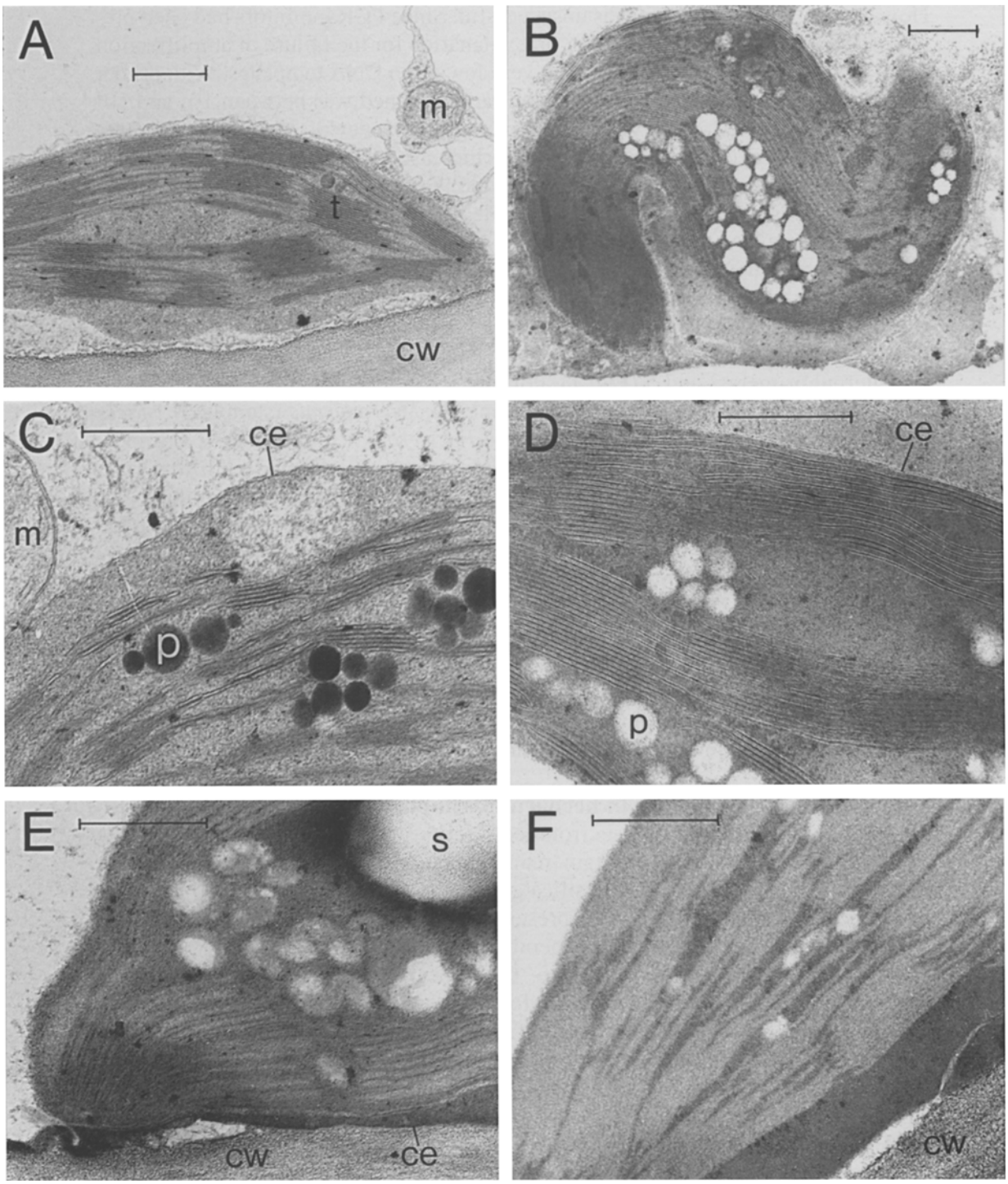

Fig. 3. Electron micrographs of chloroplast in sections from *Ilex aquifolium* leaves: chloroplasts from fresh leaves in *A* and *C*, from silica gel-dried leaves in *B* and *D*, in leaves from a 10-year-old herbarium specimen in *E*, and from a 68-year-old plant in *F*. *m* Mitochondrion, *cw* cell wall, *ce* chloroplast envelope, *p* plastoglobuli, *s* starch grain, *t* thylakoids, bars: 0.5 µm

Discussion

Out of the 17 herbarium DNA extracts in which inhibiting activities were removed and whose PCR reactions were optimized, only eight were found to contain amplifiable cpDNA. In six samples a 900 pb DNA fragment was amplified, and in the the two remaining specimens a fragment of only 369 bp was successfully amplified. These DNA fragments were sequenced and the obtained DNA sequences were compared with those kept in DNA databanks. In several cases, particularly when material and solutions were not decontaminated through exposure to UV light, contamination was identified by 100% identity with unrelated taxa. The amplification products reported here do not have more than 88% identity with any sequence present in databanks. Furthermore, these sequences fit with the expected phylogenetic relationships. Contamination is a major problem when double-PCR are carried out, and the identification of a new sequence supported by the expected phylogenetic relationships may be considered as evidence of success.
Our results show that there is no apparent correlation between the age of a herbarium sample and the success of DNA amplification, as our positive herbarium samples were collected up to 109 years ago. The conservation of amplifiable DNA in herbarium specimens seems to depend on several factors. These factors may be species-specific and related to the extreme diversity of the plant cell chemistry, and consequently are not predictable. Other factors may be related to the physiological state of the plant when it was collected, or to the mode of its preparation.

Contrary to plant tissue preserved in chemicals, high-grade DNA seems to be protected when the plant is dried (Doyle & Dickinson 1987, Sytsma & al. 1993, Pyle & Adams 1989, Thomson & Henry 1993, Harris 1993). However, the most important requirement for good preservation and further amplification of DNA is a rapid drying procedure. If the drying period is long, the plant, which is still alive, is subject to the extreme water stress, shortage of nutrients and wounding for hours or even days. It is well known that such injuries very rapidly induce phenolic compounds and free radicals production, which may influence DNA extraction and/or amplification. These metabolic and cellular responses to herbarium preparation are similar to those due to senescence. These by-products such as free radicals may have a strong influence on the cellular environment and consequently on the conservation of DNA quality (McKersie & al. 1988). Moreover, a decrease in DNA content is generally observed during early tissue senescence, which may account for as much as 20% of the total DNA (Scott & Possingham 1983).

An initial, rapid desiccation is of primary importance so as to limit the extent of senescence processes. This is the reason for the success of silica gel-dried tissues for DNA analysis (Chase & Hills 1991). Moreover, the breaking of cellular compartments during the drying stage may liberate nucleases producing endogenous hydrolytic damage. In general, herbarium samples are not dried so rapidly than in silica-gel. This is particularly true for old herbarium specimens, or plants collected in the tropics. Some plant, such as grasses or small herbs, are easy to dry, but others, e.g., succulents or hard-leaved species with a thick foliar cuticle, e.g., *Ilex*, take longer to dry, thus giving time for cellular alteration. Our electron microscope study shows a good correlation between the state of degradation

of the chloroplast membranes and the success of DNA extraction and amplification.

LINDAHL (1993) reported some observations on the preservation of DNA in solution over a long period. However, there is little literature about the modifications of DNA over long time periods in herbarium specimens. Dried herbarium tissues remain subject to oxidative damages, resulting in the alteration of the pyrimidine bases and sugar (EGLINGTON & LOGAN 1991). A reported peculiarity also observed here is the poor conservation of DNA extracts from herbarium sheets (CANO & POINAR 1993). This may be the consequence of interactions of some herbarium-specific chemical components co-extracted with the DNA. Thus, the oxidative DNA damages occurring during herbarium specimen preparation, which rapidly plateaus off (PÄÄBO 1989), may be reactivated during the extraction procedures. Furthermore, some chemicals used in herbarium disinfection or collection could interfere with DNA extraction and amplification as was demonstrated for chemotaxonomic studies by CORADIN & GIANNASI (1980).

Most of the above-mentioned features may not be under control. However, the extraction and amplification procedures may be optimized in order to overcome the low quality and/or quantity of the DNA remaining in herbarium specimens. Experiments with PCR cross-reactions have shown the presence of herbarium-specific PCR inhibitors also observed in fossil plants (GOLENBERG 1991). It is possible to reduce these inhibitors by using inhibitor-binding substances (e.g., polyvinylpyrrolidone, gelatin, BSA), by purifying the extracted DNA (e.g., silica particles or Amicon membranes), or by diluting the DNA extracts. However, such procedures were not successful with all samples.

When the PCR-inhibitor problem is solved, the extremely low amount of amplifiable DNA in some samples (less than 100–1000 molecules) is problematic. In some cases, double PCR runs using internal primers were successful. When the average size of the remaining DNA is low, amplification of smaller fragments has indeed produced positive results in previously negative trials for a larger fragment.

To summarize, herbarium specimens could be used in DNA studies, but this is far from being routine. This analysis of the reasons for amplification failures (presence of different PCR inhibiting activities and/or lack of DNA templates) shows that, in some instances, such failures could be overcome. Sampling of several samples of the same taxon is desirable. Our experience shows that it is easier to obtain herbarium specimens of plants from different parts of the World than the living material. Finally, success of DNA amplification from herbarium samples depends on so many factors (such as chemical particularities of the species, developmental stage of the collected tissue, drying method, duration and conditions of preparation) that it is not possible to outline general rules.

The authors thank the curators of the Herbarium of Geneva (G) for providing plant material. We also thank P. TABERLET and L. GIELLY (University of Grenoble, France) for their assistance in one of the extraction procedures, S. PERNIN for drawing the figures, S. DE MARCHI for technical assistance, A. STORK and the anonymous reviewers for revising the manuscript. This work was partially funded by the Fonds National Suisse de la Recherche Scientifique (grant No 31-28757.90).

References

ADAMS, R. P., MILLER, J. S., GOLENBERG, E. M., ADAMS, J. E., 1994: Conservation of plant genes II: utilization of ancient and modern DNA. – St. Louis: Missouri Botanical Garden.

GANO, R. J., POINAR, H. N., 1993: Rapid isolation of DNA from fossil and museum specimens suitable for PCR. – Biotechniques **15**: 432–435.

– – PIENIAZEK, N. J., ACRA, A., POINAR JR., G. O., 1993: Amplification and sequencing of DNA from a 120–135-million-year-old weevil. – Nature **363**: 536–538.

CARTER, M. J., MILTON, I. D., 1993: An inexpensive and simple method for DNA purifications on silica particles. – Nucl. Acids Res. **21**: 1044.

CHASE, M. W., HILLS, H. H., 1991: Silica gel: an ideal material for field preservation of leaf samples for DNA studies. – Taxon **40**: 215–220.

– SOLTIS, D. E., OLMSTEAD, R. G., MORGAN, D., LES, D. H., MISHLER, B. D., DUVALL, M. R., PRICE, R. A., HILLS, H. G., QIU, Y. L., KRON, K. A., RETTIG, J. H., CONTI, E., PALMER, J. D., MANHART, J. R., SYTSMA, K. J., MICHAELS, H. J., KRESS, W. J., KAROL, K. G., KLARK, W. D., HEDREN, M., GAUT, B. S., JANSEN, R. K., KIM, K. J., WIMPEE, C. F., SMITH, J. F., FURNIER, G. R., STRAUSS, S. H., XIANG, Q. Y., PLUNKETT, S. E., SOLTIS, P. S., SWENSEN, S. M., WILLIAMS, S. E., GADEK, P. A., QUINN, C. J., EGUIARTE, L. E., GOLENBERG, E. M., LEARN JR., G. H., GRAHAM, S. W., BARRETT, S. C., DAYANANDAN, S., ALBERT, V. A., 1993: Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbc*L. – Ann. Missouri Bot. Gard. **80**: 528–580.

COOPER, A., MOURER-CHAUVIRE, C., CHAMBERS, G. K., VON HAESELER, A., WILSON, A. C., PÄÄBO, S., 1992: Independent origin of New Zealand moas and kiwis. – Proc. Natl. Acad. Sci. USA **89**: 8741–8744.

CORADIN, L., GIANNASI, D. L., 1980: The effects of chemical preservations on plant collections to be used in chemotaxonomic surveys. – Taxon **29**: 33–40.

DESALLE, R., GATESY, J., WHEELER, W., GRIMALDI, D., 1992: DNA sequences from a fossil termite in Oligo-Miocene amber and their phylogenetic implications. – Science **257**: 1933–1936.

DOYLE, J. J., DICKSON, E. E., 1987: Preservation of plant samples for DNA restriction endonuclease analysis. – Taxon **36**: 715–722.

– DOYLE, J. L., 1990: Isolation of plant DNA from fresh tissue. – Focus **12**: 13–15.

EGLINGTON, G., LOGAN, G. A., 1991: Molecular preservation. – Philos. Trans. Roy. Soc. London B **333**: 315–328.

GOLENBERG, E. M., 1991: Amplification and analysis of Miocene plant fossil DNA. – Philos. Trans. Roy. Soc. London B **333**: 419–427.

– GIANNASI, D. E., CLEGG, M. T., SMILEY, C. J., DURBIN, M., HENDERSON, D., ZURAWSKI, G., 1990: Chloroplast DNA sequence from a Miocene *Magnolia* species. – Nature **344**: 656–658.

GUSTINCICH, S., MANFIOLETTI, G., DEL SAL, G., SCHNEIDER, C., CARNINCI, P., 1991: A fast method for high-quality genomic DNA extraction from whole human blood. – Biotechniques **11**: 298–302.

HAGELBERG, E., CLEGG, J. B., 1993: Genetic polymorphism in prehistoric pacific islanders determined by analysis of ancient bone DNA. – Proc. Roy Soc. London B **252**: 163–170.

HARRIS, S. A., 1993: DNA analysis of tropical plant species: an assessment of different drying methods. – Pl. Syst. Evol. **188**: 57–64.

HÖSS, M., PÄÄBO, S., 1993: DNA extraction from pleistocene bones by silica-based purification method. – Nucl. Acids Res. **21**: 3913–3914.

JANCZEWSKI, D. J., YUHKI, N., GILBERT, D. A., JEFFERSON, G. T., O'BRIEN, S. J., 1992: Molecular phylogenetic inference from sabre-toothed cat fossils of Rancho La Brea. – Proc. Natl. Acad. Sci. USA **89**: 9769–9773.

LEWIN, R., 1994: Fact, fiction and fossil DNA. – New Scientist **29**: 38–41.

LINDAHL, T., 1993: Instability and decay of the primary structure of DNA. – Nature **362**: 709–715.

LOOCKERMANN, D. J., JANSEN, R. K., 1995: The use of herbarium material for DNA studies. – In STUESSY, T. F., (Ed.): Sampling the green world. – Columbia Univ. Press. (In press.)
LOOMIS, W. D., BATAILLE, J., 1966: Plant phenolic compounds and the isolation of plant enzymes. – Phytochemistry **5**: 423–438.
MCKERSIE, B. D., SENARATNA, T., WALKER, M. A., KENDALL, E. J., HETHERINGTON, P. R., 1988: Deterioration of the membrane during aging in plants: evidence for free radical mediation. – In NOODEON, L. D., LEOPOLD, A. C., (Eds): Senescence and aging in plants, pp. 441–464. – San Diego: Academic Press.
PÄÄBO, S., 1989: Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. – Proc. Natl. Acad. Sci. USA **86**: 1939–1943.
– 1991: Amplifying DNA from archeological remains: a meeting report. – PCR Methods & Protocols **1**: 107–110.
– HIGUSCHI, R. G., WILSON, A. C., 1989: Ancient DNA and the polymerase chain reaction. – J. Biol. Chem. **264**: 9709–9712.
PYLE, M. M., ADAMS, R. P., 1989: In situ preservation of DNA in plant specimens. – Taxon **38**: 576–581.
REYNOLDS, R. S., 1963: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. – J. Cell Biol. **17**: 208–212.
SARKAR, G., SOMMER, S. S., 1990: Shedding light on PCR contamination. – Nature **343**: 27.
SAVOLAINEN, V., MANNEN, J. F., DOUZERY, E., SPICHIGER, R., 1994: Molecular phylogeny of families related to *Celastrales* based on rbcL 5′ flanking sequences. – Mol. Phyl. Evol. **3**: 27–37.
SCOTT, N. E., POSSINGHAM, J. V., 1983: Changes in chloroplast DNA levels during growth of spinach leaves. – J. Exp. Bot. **34**: 1756–1767.
SOLTIS, P. S., SOLTIS, D. E., SMILEY, C. J., 1992: An rbcL sequence from a Miocene *Taxodium* (bald cypress). – Proc. Natl. Acad. Sci. USA **89**: 449–451.
SPICHIGER, R., SAVOLAINEN, V., MANEN, J. F., 1993: Systematic affinities of *Aquifoliaceae* and *Icacinaceae* from molecular data analysis. – Candollea **48**: 459–464.
SYTSMA, K. J., GIVNISH, T. J., SMITH, J. F., HAHN, W. J., 1993: Collection and storage of land plant samples for macromolecular comparisons. – Methods Enzymol. **224**: 23–37.
TABERLET, P., GIELLY, L., PAUTOU, G., BOUVET, J., 1991: Universal primers for amplification of three non-coding regions of chloroplast DNA. – Pl. Mol. Biol. **17**: 1105–1109.
TAYLOR, J. W., SWANN, E. C., 1994: DNA from herbarium specimens. – In HERRMANN, B., HUMMEL, S., (Eds): Ancient DNA, pp. 167–181. – Berlin: Springer.
THOMSON, D., HENRY, R., 1993: Use of DNA from dry leaves for PCR and RAPD analysis. – Pl. Mol. Biol. Rep. **11**: 202–206.
WEBB, D. M., KNAPP, S. J., 1990: DNA extraction from previously recalcitrant plant genus. – Pl. Mol. Biol. **8**: 180–185.
WEBER, A. W., 1982: Mnemonic three-letter acronyms for the families of vascular plants: a device for more effective herbarium curation. – Taxon **31**: 74–88.

Addresses of the authors: RODOLPHE SPICHIGER, JEAN-FRANÇOIS MANEN, VINCENT SAVOLAINEN (correspondence), and PHILIPPE CUÉNOUD, Conservatoire et Jardin Botaniques de Genéve, CH-1292 Chambésy, Geneva, Switzerland, phone number (+41 22) 732 69 69, fax number (+41 22) 738 45 97, e-mail savolainen@cjb.unige.ch. – MARIA D. P. MARTINEZ, Conservatoire et Jardin Botaniques de Genève, CH-1292 Chambésy, Geneva, Switzerland and Centro de Estudios Farmacológicos y Botánicos (CEFyBO), CONICET, RA-1414 Buenos Aires, Argentina. – MICHÈLE CRÈVECOEUR, Laboratoire de Biochimie et Physiologie Végétales, Université de Genève, CH-1205 Geneva, Switzerland.

Accepted February 22, 1995 by F. EHRENDORFER