Short Technical Reports

Direct Amplification of the Entire ITS Region from Poorly Preserved Plant Material Using Recombinant PCR

BioTechniques 27:1180-1186 (December 1999)

ABSTRACT

Sequences of the internal transcribed spacers (ITS) of the nuclear ribosomal DNA are important molecular markers in phylogenetic analyses. To obtain sequences from herbarium material in which DNA often is severely degraded, the ITS region has to be amplified in two steps. Two methods that reduce bench time and reagents used are described. (i) *Separately amplified preparations of subunits ITS-1 and ITS-2 are combined before purification. The presence of two fragments in the sequencing reaction does not impair the quality of sequences.* (ii) *Newly designed internal primers amplify partly overlapping regions of the two subunits. A combination of these internal primers with the external primers in one PCR allows the amplification of the entire ITS region even when degraded DNAs are used. This recombinant PCR approach, taking into account the +A bases added by several* Taq *DNA polymerases, will also be useful with other marker regions used in molecular phylogenetics.*

INTRODUCTION

The advent of DNA-based markers in phylogenetic reconstruction provided researchers with a broad range of tools to study biological diversity and evolution from the population level up to the comparison of families and even kingdoms (16). In particular, the use of PCR allows the analysis of minute amounts of DNA (24) and thus opens the collections in museums to investigation (14,19). The success of amplification and analysis mostly depends on the preservation of the DNA and on the purification of the DNA extracted (6). Slow drying of biological material or storage under unfavorable conditions often results in severely degraded DNA (5,10), which precludes the amplification of fragments longer than about 150–400 bp. Although good preservation methods are available for newly collected material (1,7,11,22), the analysis of older samples often is possible only through stepwise amplification and the sequencing of short, overlapping fragments of the desired DNA region, sometimes a time-consuming and laborious task.

Various methods have described how to circumvent problems arising from fragmented DNA by introducing a repair step before PCR amplification (5,21) or during the first rounds of amplification of the desired region (5). This often is the only way to obtain sequence information when DNA is old and highly fragmented (e.g., fossil DNA). In cases in which the amplification of DNA stretches of approximately 300 bp length is possible (as in well preserved herbarium material), a modified PCR protocol can spare the additional repair steps mentioned above (5,21). These modifications are summarized in the following sections with examples from the internal transcribed spacers (ITS) region of plants.

The nuclear ribosomal DNA (nrDNA) (Figure 1) consists of three coding subunits (18S-, 5.8S- and 26S-subunits in plants) that are separated by ITS-1 and ITS-2. The tandemly repeated rDNA occurs in high copy number in the genome. The two spacers are part of the transcriptional unit, but are not included in the final ribosomal RNA. They appear to serve in maturation of the primary transcripts by bringing the subunit boundaries closer, thus allowing the three subunits to be processed (18). Accordingly, core parts of the spacer sequences, which are essential for obtaining the secondary structure, are evolutionarily conserved within green plants (9,13,15).

Other parts of the two spacers show considerable variation in nucleotide comparisons among species. This fact, together with features such as conserved flanking regions that allow the construction of universal primers (23), rapid concerted evolution, promoting intragenomic uniformity of the copies and a sequence length of approximately 700 bp (suitable for PCR amplification of the entire ITS region, including 5.8S rDNA), makes the ITS one of the most attractive nuclear markers for phylogenetic analyses at lower systematic ranks (2,3).

Even though the amplification of the entire ITS region from fresh or well-preserved plant material is generally easy, severely degraded DNA necessitates two separate amplification reactions to cover the region. Therefore, internal primers that anneal in the conserved 5.8S rDNA between the two spacers are used. Combining each of the external primers with the internal primer of the complementary strand amplifies two subunits, each approximately 350 bp in length. This procedure normally results in excellent sequences, but doubles the amount of bench time and reagents needed because ITS-1 and ITS-2 must be processed and sequenced separately. Two approaches that reduce sample handling are described. A first test showed that the combination of separately amplified ITS-1 and ITS-2 fragments for purification and sequencing did not impair results. In a second approach, a recombinant PCR method (17) describes how to obtain full-length sequences of the entire ITS region in one step, using newly developed internal primers.

MATERIALS AND METHODS

Genomic DNA was extracted from up to 50 mg dried leaves with the DNeasy™ Plant Kit (Qiagen, Düren, Germany) after slightly modifying the manufacturer's instructions, as described by Blattner and Kadereit (4). The methods described here were developed and tested using species from various angiosperm families such as Euphorbiaceae, Lamiaceae, Papaveraceae, Poaceae and Malvaceae.

The ITS region usually was PCR amplified using the two primers ITS-A and ITS-B (Table 1), in 50 µL reaction volume. PCR conditions were carried out in a GeneAmp® PCR System 9700 Thermal Cycler (PE Biosystems, Foster City, CA, USA) as follows. An initial denaturation at 95°C for 2 min was followed by 30 cycles of annealing at

Table 1. Sequences of the PCR Primer Mentioned in the Text

Primer	Sequence
ITS-A	5′-GGAAGGAGAAGTCGTAACAAGG-3′
ITS-B	5′-CTTTTCCTCCGCTTATTGATATG-3′
ITS-C	5′-GCAATTCACACCAAGTATCGC-3′
ITS-D	5′-CTCTCGGCAACGGATATCTCG-3′
ITS-E	5′-CGGCAACGGATATCTCGGCTC-3′

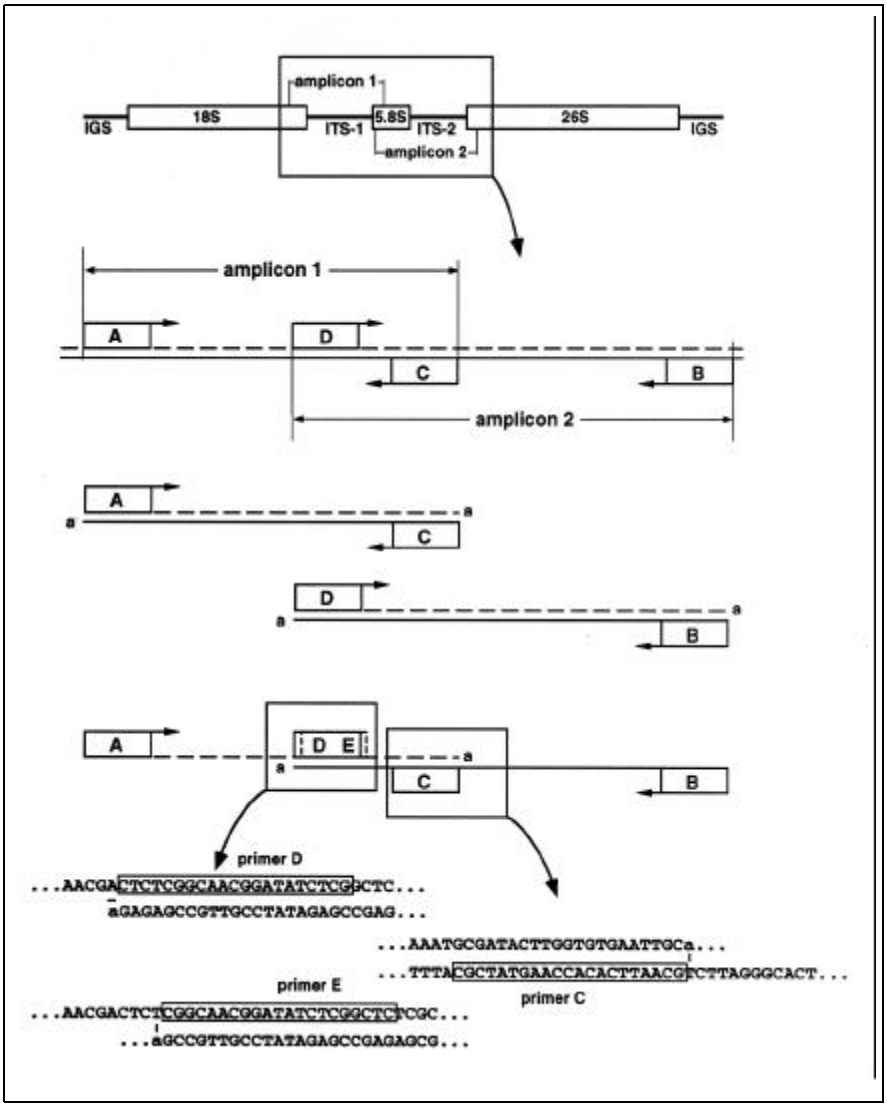

Figure 1. Schematic representation of a nrDNA repeat unit (top) and self-annealing of overlapping parts of separately amplified ITS subunits (below). IGS depicts the intergenic spacer separating the rDNA repeats. Primer positions are given with A, B, C, D and E; protruding +A bases, attached by *Taq* DNA polymerases to the 3′ end of the strand, are marked by "a". The priming sites (boxed) of the internal primers C, D and E are shown in more detail. | represents the possibility of basepairing at the end of the amplicons, whereas mispriming is represented by "-".

55°C for 30 s, extension at 70°C for 1 min and denaturation at 95°C for 20 s, with a final extension at 70°C for 7 min. Amplification was carried out with 1 U *Taq* DNA Polymerase (Roche Molecular Biochemical, Mannheim, Germany) in the supplied reaction buffer, 0.2 mM of each dNTP, 50 pmol of each primer and 10–25 ng of total DNA. After amplification, the PCR products were cleaned with the QIAquick™ PCR Purification Kit (Qiagen). When degraded template DNA was used, ITS-1 and ITS-2 were amplified separately using the primer combinations of ITS-A with ITS-C, and ITS-B with ITS-D, using identical ampliication conditions as above. Using primers ITS-C and ITS-D instead of White et al. (23) ITS2 and ITS3 results in a 72 bp overlap at the beginning of the 5.8S rDNA (corresponding to 5.8S rDNA alignment positions 6–78 in Reference 8).

PCRs of the separately amplified spacers were pooled and cleaned with the QIAquick PCR purification kit before sequencing. After checking DNA concentration on a 1.8% agarose gel, approximately 40 ng PCR product (2 × 40 ng in pooled reactions) were used in a 10 µL cycle sequencing reaction with the BigDye™ Terminator Kit (PE Biosystems) according to the manufacturer's instructions. ITS-A and ITS-B were used as sequencing primers. Sequencing reactions were analyzed on a Model 377 DNA Sequencer (PE Biosystems), in forward and reverse sequences and then combined in a consensus sequence.

To amplify the entire ITS region in a single step even when badly preserved DNA was present as the template, I used a modified primer, ITS-D. A 4 bp shift of the primer position toward ITS-2 resulted in a primer sequence (ITS-E), which is next to a T at the 5′ end (Figure 1). The additional A added by most *Taq* DNA polymerases to the elongated strand thus matches the sequence of the 5.8S rDNA. In amplification reactions (contents above), primers ITS-A and ITS-B were supplemented by 1 pmol of each primer, ITS-C and ITS-E. PCR was carried out using the following program. An initial denaturation at 95°C for 2 min was followed by 10 cycles of annealing at 55°C for 30 s, extension at 70°C for 30 s and denaturation at 95°C for 20 s. This was followed by 32 cycles of annealing at 55°C for 30 s, extension at 70°C for 1 min, denaturation at 95°C for 20 s and a final extension at 70°C for 7 min. The overlapping parts of the two spacer regions served as priming sites for subsequent PCR cycles, when primers ITS-C and ITS-E are exhausted in the reaction mixture.

RESULTS AND DISCUSSION

Sequences obtained by the combination of separately amplified ITS-1 and ITS-2 amplicons in purification and sequencing were clearly readable, and no negative effects that could result from this procedure were observed. Additionally, nearly complete sequences of the entire ITS region could be obtained, when ITS-1 was sequenced with the forward primer (ITS-A). A signal decrease of approximately 50% in the sequence chromatogram (Figure 2) marks the end of amplicon 1 (ITS-A to ITS-C), but did not prevent further sequence reading. Sequences that exceeded the amplicon border could not be observed in the reverse sequencing reactions, where the signal dropped to background level at the end of amplicon 2.

A closer investigation of the overlapping ends of the two amplicons is necessary to explain this different behavior. The main difference between the two ends is a T that appears adjacent to primer ITS-C and primer ITS-D next to an A (Figure 1). Thus, protruding +A bases, eventually attached by the *Taq* DNA polymerase to the 3′ end of newly synthesized DNA strands, result in a matching amplicon end in amplicon 1. In the sequencing reaction, the forward strands of amplicon 1 will anneal to the overlapping part of amplicon 2 and serve as a starting point for strand elongation, no matter whether a +A base occurs or not. In amplicon 2, where +A bases result in mismatches, the elongation reaction will only start when no A is attached to the end. This occurs rarely and explains the severe signal decrease observed in reverse sequencing reactions.

The frequently used internal primers designed by White et al. (23) allow no

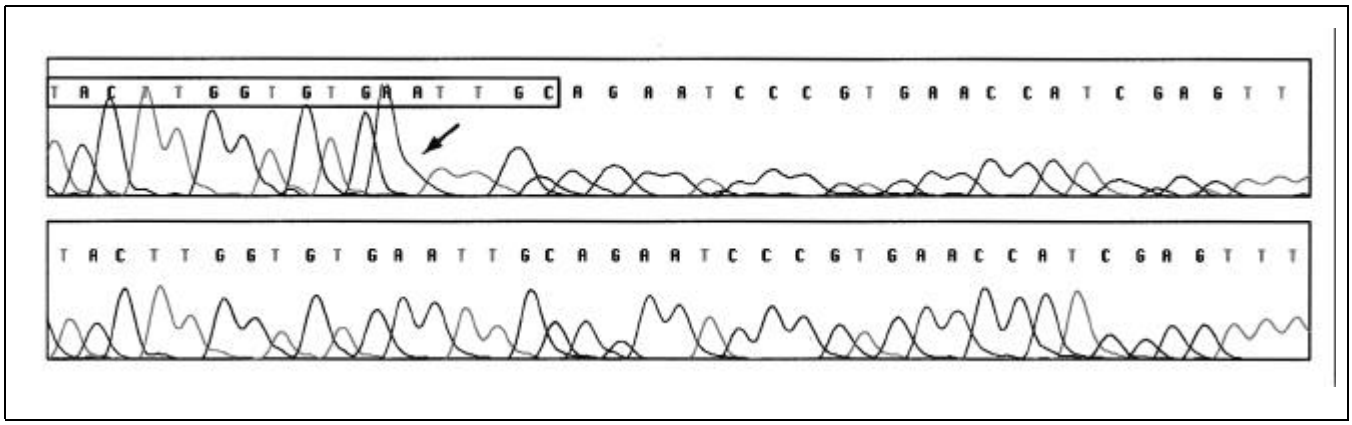

Figure 2. Sequence electropherograms showing the 3′ end of amplicon 1 in ITS sequences of *Stylophorum diphyllum* (Papaveraceae) obtained from combined amplification products (top) and from a single amplification of the entire ITS region. The separately amplified subunits of the ITS region were pooled before purification and were both present in the sequencing reaction. The end of the annealing site of primer ITS-C is boxed. Clearly visible is a signal reduction (arrow) near the end of the amplicon 1 (primer + A), which did not circumvent sequence detection for nearly the entire amplicon 2.

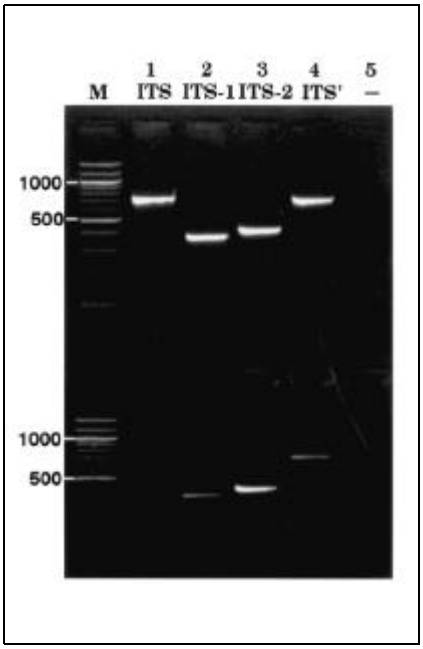

Figure 3. Comparison between ITS amplifications starting with well-preserved DNA (top) and degraded DNA (bottom). In the PCRs separated in the upper part of the gel, DNA extracted from rapidly dried leaves of *Macaranga petanostyla* (Euphorbiaceae) was used. The lower part shows fragments amplified from severely degraded DNA of *M. aleuritoides*. In lane 1, PCR products are amplified with primers ITS-A and ITS-B, resulting in the entire ITS region when good template DNA is used. Lanes 2 and 3 contain ITS-1 (primers A and C) and ITS-2 (primers B and E), respectively. In lane 4, PCR products that have been amplified with a reaction mixture containing all four primers in a standard PCR protocol (30 cycles) are shown.

strand elongation during sequencing because they are neighbored by G and C, respectively. This means that a combination of separately amplified ITS-1 and ITS-2 before purification is possible, but will not result in strand elongation during the sequencing reaction.

In the second approach, the newly designed primer ITS-E behaves comparably to ITS-C in promoting strand elongation into adjacent regions, even when +A bases occur (Figure 1). The combination of minute amounts of primers C and E, together with the external primers ITS-A and ITS-B in PCR, resulted in full-length ITS products when degraded template DNA was used (Figure 3). This shows that amplicons 1 and 2, which were amplified during the initial PCR cycles, serve as a template for the amplification of the entire ITS region, at least after primers C and E were exhausted. The product can be sequenced like ITS amplicons that are derived from fresh DNA, even if less DNA is obtained when starting from fragmented templates.

The described method makes use of DNA fragments being able to serve as starting points for *Taq* DNA polymerase in PCR when overlapping parts of fragments exist. Generally, this should occur during every PCR cycle, theoretically allowing self-assemblage of highly degraded DNA during PCR (5). In practice, however, if amplicons are obtained at all (5), they are rarely suitable for sequencing. Mostly, they are an accumu-

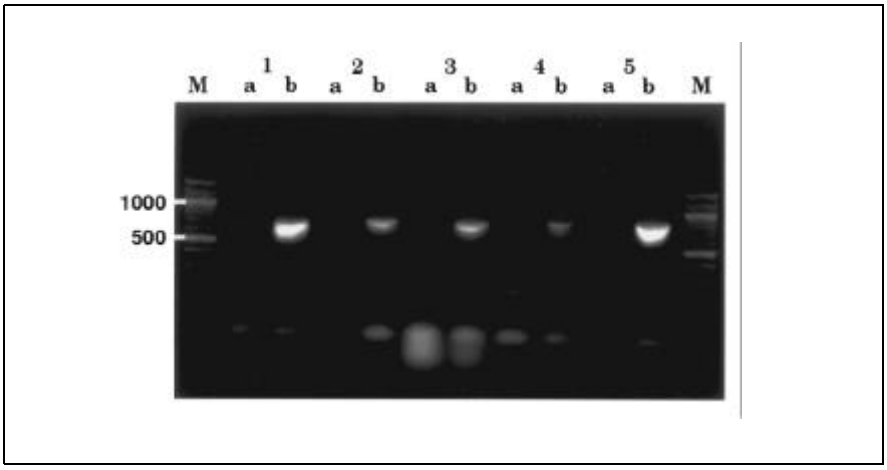

Figure 4. Comparison of PCRs conducted with two primers (primers A and B) and with four primers (primers A, B, C and E) from degraded template DNAs. The taxa used were 1, *Hordeum vulgare* (Poaceae); 2, *Milula spicata* (Alliaceae); 3, *Papaver persicum* (Papaveraceae); 4, *Helmiopsis pseudo-populus* (Malvaceae) and 5, *Potentilla cinerea* (Rosaceae).

lation of products with small length differences or multiple overlapping sequences that may result from mispriming at small sequence homologies interspersed within the template DNA (jumping PCR). The approach described here allows amplification of the two partial ITS sequences during the initial PCR cycles; it shifts later on, after primers C and E are exhausted, to an amplification mode where the overlapping parts of amplicon 1 and 2 serve as priming sites, together with primers A and B, respectively. After the production of the first full-length products, these later serve as template for a "regular" ITS amplification.

The use of amplicon 1 (A–C) together with amplicon 2 (B–E) in a combined purification step is not recommended. In that case, both ends of the overlapping part of the 5.8S rDNA serve as starting points for strand elongation during the sequencing reaction. In the sequencing gel, where fragments are separated by length, two parallel sequences will occur. The additional sequence will start at the base position that corresponds to the length of the second amplicon present in the reaction.

In summary, there are several methods to choose from when DNA isolated from herbarium material is to be used for ITS amplification. Usually ITS-1 and ITS-2 are separately amplified, purified and sequenced. This protocol can be speeded up when the amplified PCR products are pooled and purified in one step. Neither PCR products amplified with the White et al. (23) internal primers nor with primers ITS-C/ITS-D impaired the quality of sequences obtained when both amplicons were present in the sequencing reaction.

Alternatively, all four primers (A, B, C and E) can be used in one PCR, which results in full-length ITS products. Using this protocol as a standard approach will speed up amplification when DNAs in differently preserved states have to be analyzed. The two additional primers, present only in minute amounts, do not influence PCR with undegraded DNA, but allow the start of the chain reaction when poorly preserved DNA is used. This method allows the analysis of forward and reverse strands with only two sequencing reactions, even when degraded templates are used. When DNA degradation is quite high and does not result in separate ITS-1 and ITS-2 amplification products, an additional reconstruction step (5,21) might result in PCR products.

The newly developed internal primers, C and E, were designed to work well in land plants, and could also be useful in algae or fungi. In any case, it would be advantageous to first check the primer annealing sites of the internal primers. This step can be carried out either by sequencing the entire ITS

region starting from undegraded DNA or in an alignment of 5.8S rDNAs downloaded from a DNA database that contains representatives of the group under study (8). Database comparisons will also allow users to design primers that discriminate against fungal sequences. These often cause severe problems in ITS analyses of plants that are inhabited by fungi (12,25) or when badly conserved herbarium sheets are used for DNA extraction.

Taking into account the +A bases added by several *Taq* DNA polymerases, primers designed to promote self-priming of overlapping parts of short fragments of a longer target DNA can be useful in a wide variety of marker genes used in molecular phylogenetics.

REFERENCES

1. **Adams, R.P., N. Do and C. Ge-lin.** 1991. Preservation of DNA in plant specimens from tropical species by desiccation, p. 135-152. *In* R.P. Adams and J.E. Adams (Eds.), Conservation of Plant Genes: DNA Banking and In Vitro Biotechnology. Academic Press, San Diego.
2. **Baldwin, B.G.** 1992. Phylogenetic utility of the internal transcribed spacers of the ribosomal DNA in plants: an example from the compositae. Mol. Phylogenet. Evol. *1*:3-16.
3. **Baldwin, B.G., M.J. Sanderson, J.M. Porter, M.F. Wojciechowski, C.S. Campbell and M.J. Donoghue.** 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Missouri Bot. Gard. *82*:247-277.
4. **Blattner, F.R. and J.W. Kadereit.** 1999. Morphological evolution and ecological diversification of the forest-dwelling poppies (*Papaveraceae: Chelidonioideae*) as deduced from a molecular phylogeny of the ITS region. Plant Syst. Evol. *218*:(4).
5. **Golenberg, E.M., A. Bickel and P. Weihs.** 1996. Effect of highly fragmented DNA on PCR. Nucleic Acids Res. *24*:5026-5033.
6. **Hänni, C., T. Brousseau, V. Laudet and D. Stehelin.** 1995. Isopropanol precipitation removes PCR inhibitors from ancient bone extracts. Nucleic Acids Res. *23*:881-882.
7. **Harris, S.A.** 1993. DNA analysis of tropical plant species: an assessment of different drying methods. Plant Syst. Evol. *188*:57-64.
8. **Hershkovitz, M.A. and L.A. Lewis.** 1996. Deep-level diagnostic value of the rDNA-ITS region. Mol. Biol. Evol. *13*:1276-1295.
9. **Hershkovitz, M.A. and E.A. Zimmer.** 1996. Conservation patterns in angiosperm rDNA ITS2 sequences. Nucleic Acids Res. *24*:2857-2867.
10. **Höss, M., P. Jaruga, T.H. Zastawny, M. Dizdaroglu and S. Pääbo.** 1996. DNA damage and DNA sequence retrievel from ancient tissues. Nucleic Acids Res. *24*:1304-1307.
11. **Liston, A., L.D. Rieseberg, R.P. Adams, N. Do and G. Zhu.** 1990. A method for collecting dried plant specimens for DNA and isozyme analyses, and the result of a field test in Xinjiang, China. Ann. Missouri Bot. Gard. *77*:859-863.
12. **Liston, A., W.A. Robinson, J.M. Oliphant and E.R. Alvarez-Buylla.** 1996. Length variation in the nuclear ribosomal DNA internal transcribed spacer region of non-flowering seed plants. Syst. Bot. *21*:109-120.
13. **Liu, J.-S. and C.L. Schardl.** 1994. A conserved sequence in internal transcribed spacer 1 of plant nuclear rRNA genes. Plant Mol. Biol. *26*:775-778.
14. **Loockerman, D.J. and R.K. Jansen.** 1996. The use of herbarium material for DNA studies, p. 205-220. *In* T.F. Stuessy and S.H. Sohmer (Eds.), Sampling the Green World. Columbia University Press, New York.
15. **Mai, J.C. and A.W. Coleman.** 1997. The internal transcribed spacer 2 exhibits a common secondary structure in green algae and flowering plants. J. Mol. Evol. *44*:258-271.
16. **Moritz, C. and D.M. Hillis.** 1996. Molecular systematics: context and controversies, p. 1-13. *In* D.M. Hillis, C. Moritz and B.K. Mable (Eds.), Molecular Systematics, 2nd ed. Sinauer Assoc., Sunderland, MA.
17. **Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn and E. Erlich.** 1986. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb. Symp. *51*:263-273.
18. **Musters, W., K. Boon, C.A.F.M. van der Sande, H. van Heerikhuizen and R.J. Planta.** 1990. Functional analysis of transcribed spacers of yeast ribosomal DNA. EMBO J. *9*:3989-3996.
19. **Pääbo, S.** 1990. Amplifying ancient DNA, p. 159-166. *In* M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego.
20. **Pääbo, S., R.G. Higuchi and A.C. Wilson.** 1989. Ancient DNA and the polymerase chain reaction. The emerging field of molecular archaeology. J. Biol. Chem. *264*:9709-9712.
21. **Pusch, C.M., I. Giddings and M. Scholz.** 1998. Repair of degraded duplex DNA from prehistoric samples using *Escherichia coli* DNA polymerase I and T4 DNA ligase. Nucleic Acids Res. *26*:857-859.
22. **Sytsma, K.J., T.J. Givnish, J.F. Smith and W.J. Hahn.** 1993. Collection and storage of land plant samples for macromolecular comparisons. Methods Enzymol. *224*:23-37.
23. **White, T.J., T. Bruns, S. Lee and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315-322. *In* M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego.
24. **Wolfe, A.D. and A. Liston.** 1998. Contributions of PCR-based methods to plant systematics and evolutionary biology, p. 43-86. *In* D.E. Soltis, P.S. Soltis and J.J. Doyle (Eds.), Molecular Systematics of Plants II: DNA Sequencing. Kluwer Acad. Publ., Dordrecht, NL.
25. **Zhang, W., J.F. Wendel and L.G. Clark.** 1997. Bamboozled again! Isolation of fungal rDNA sequences from bamboos (Poaceae: Bambusoideae). Mol. Phylogenet. Evol. *8*:205-217.

I would like to thank Andrea E. Schwarzbach (Bloomington) and Ivana Stehlik (Zürich) for helpful comments on the manuscript. *Address correspondence to Dr. Frank R. Blattner, Dept. of Taxonomy, Institute of Plant Genetics and Crop Plant Research (IPK), D-06466 Gatersleben, Germany. Internet: blattner@ipk-gatersleben.de*

Received 14 April 1999; accepted 19 July 1999.

Frank R. Blattner
Institute of Plant Genetics and Crop Plant Research (IPK)
Gatersleben, Germany