Improving procedures for obtaining Sanger sequences from old herbarium specimens

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Because DNA degrades over time, extracting DNA of sufficient quality for sequencing is presumed to be more difficult from older than younger herbarium specimens. Although massive parallel sequencing techniques have clear advantages when it comes to sequencing ancient DNA, Sanger sequencing is still in frequent use, prompting us to test and improve its application on herbarium specimens. During molecular phylogenetic investigations of the subfamily Lamioideae (Lamiaceae) and subgroups, we extracted DNA from 651 herbarium specimens collected between 1826 and 2006 using regular mini-prep methods. The aim was to obtain DNA of sufficient quality for Sanger sequencing of various plastid and nuclear genetic markers. Here, we report successful Sanger sequencing of the commonly used plastid marker, rps16, as a conservative measure of DNA quality, and logistic regression to investigate the relationship between age of the material and DNA quality. Our result indicates that the upper age limit for obtaining DNA suitable for Sanger sequencing from herbarium specimens using regular mini-prep DNA extraction methods has not been reached. After simple modifications to the regular DNA mini-prep and PCR procedures, at least one genetic marker was successfully sequenced for about 90 % of the specimens tested, the oldest being 168 years old. Jointly, despite the technique's drawbacks, these results demonstrate a high success rate of Sanger sequencing of herbarium specimens.

Keywords
DNA extraction, Herbarium specimens, Lamiaceae, Mini-prep, PCR, Replicates, Sanger sequencing

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Fresh, silica dried, or frozen plant tissue is ideal for obtaining DNA sequences. However, such material is often not available due to a variety of reasons, such as rarity, geographical restriction or remoteness of the taxon of interest, or even extinction. Most taxa are available, however, as preserved specimens in at least one of the World's many natural history collections. Archived scientific collections provide verifiable and unique records of the existence of an organism at a given time and place. Moreover, herbaria, fungaria, and seed-, culture-, in vitro-, tissue-, and DNA collections, often contain expert-curated specimens collected throughout the world, some of which are several 100 years old.

Herbarium specimens are relatively easily accessed due to international specimen exchange agreements and represent a great resource for biological research (e.g., Andrew et al. 2018; Bebber et al. 2010, Kohn et al. 2005) – not only for morphological investigations but also for molecular research (Bieker and Martin, 2018), provided DNA of sufficient quality for successful DNA sequencing can be obtained (hereafter referred to as ‚quality-DNA'). Botanical collections have been used in a vast number of molecular studies since the mid-80s to address questions related to phylogenetic relationships, nomenclatural identity, origin of populations, function and evolution of genes (e.g., Ames and Spooner 2008, Andreasen et al. 2009, De Castro and Menale 2004, Jankowiak et al. 2005, Lambertini et al. 2008, Rogers and

Bendich 1985), and more recently for the studies of ancient plant genomes (Bieker and Martin 2018, Kistler et al. 2020).

Because DNA degrades and becomes recalcitrant over time, obtaining ‚quality-DNA' is expected to be more difficult from older than younger plant material (Pääbo 1989). Substantial degradation of DNA is reported in investigations of old biological collections with DNA fragments usually ranging in size from 50–500 bp (Soltis and Soltis 1993). Post-mortem degradation of DNA is an inherent trait and unending process of biological materials, challenging the usability of archived biological specimens in studies on DNA (e.g., Allentoft et al. 2012). The application of PCR on such materials often requires significant modification to standard protocols (Fulton and Stiller 2012). Recently developed PCR-free high-throughput sequencing (HTS) approaches mitigates some of the challenges with recalcitrant DNA. Such HTS approaches are, however, still not available nor affordable in many labs, and in the field of phylogenetic systematics, Sanger sequencing remains a much-used technology.

For our molecular investigations of the subfamily Lamioideae and subgroups (e.g., Bendiksby et al. 2011a, b, c, 2014; Scheen et al. 2010), we extracted and PCR amplified DNA from more than 650 herbarium specimens collected between 1826 and 2006. We used regular mini-prep DNA extraction kits and standard PCR reactions (referred to hereafter as ‚the regular procedure'; Box 1) prior to Sanger sequencing several plastid and two nuclear DNA regions. Several specimens, some of which were of high importance for understanding the phylogenetic relationships, did not amplify. We therefore put effort into testing several minor modifications to the ‚regular procedure' to obtain quality-DNA from these important accessions.

The aim of this short communication is **1** to showcase how valuable herbaria are as a data source for molecular biosystematics research and **2** to present the modified procedures that made us able to obtain DNA suitable for Sanger sequencing from specimens that were previously discarded as useless for molecular studies. We show the relationship between the age of the plant material and sequencing success, using successful rps16 sequencing as a conservative measure of DNA quality. We use logistic regression to illustrate the relationship between age and DNA quality in the material.

Material and methods

We compiled information about sequencing success and age of the 651 herbarium specimens that were used in our biosystematics studies of the subfamily Lamioideae and subgroups (Bendiksby et al. 2011a, b, c, 2014; Salmaki et al. 2013, 2015; Scheen et al. 2010). For these studies, we targeted six plastid *(trn*L intron, *trn*L-*trn*F spacer, *rps*16 intron, *mat*K, *trn*S-*trn*G spacer, *psb*A-*trn*H spacer) and two nuclear (NRPA2, 5S-NTS) DNA regions for all or subsets of the specimens.

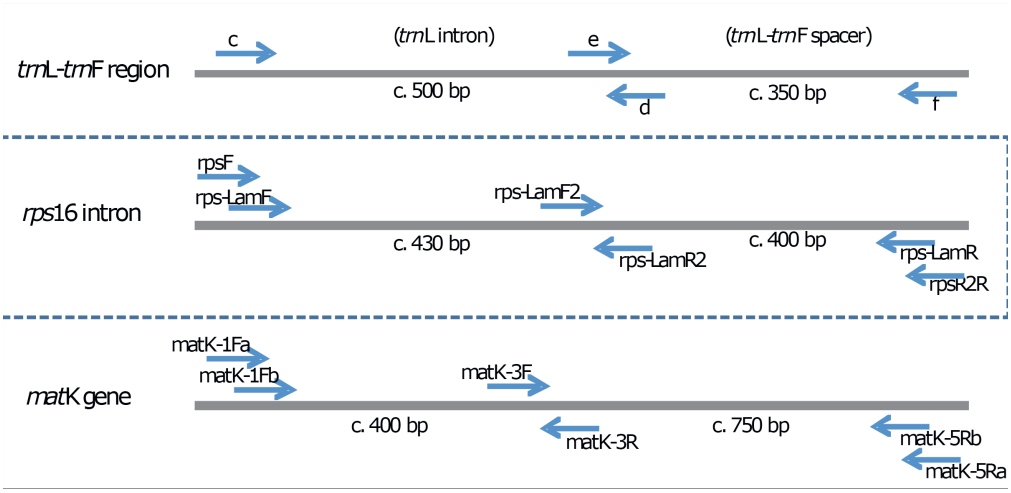

Fig. 1. Primer locations for the plastid regions amplified as one or two fragments (schematic), indicating approximate fragment lengths in base pairs (bp). The primers c, d, e, and f were published by Taberlet et al. (1991), rpsF and rpsR2R by Oxelman et al. (1997), and the remaining illustrated primers by Bendiksby et al. (2011b). Regions amplified as single fragments: *psb*A-*trn*H (c. 330 bp) using the primers psbAF and trnHR (Sang et al. 1997), *trn*S-*trn*G (c. 530 bp) using the primers trnS^{GSU} and trnG (Hamilton 1999), NRPA2 (c. 700-830 bp) using primers published by Bendiksby et al. (2011a), and 5S-NTS (c. 400 bp) using the forward primer 5S-30 (5' GGATCCCATCAGAACTCCG 3'; Bendiksby 2002) and a non-degenerate version of PII from Cox et al. (1992) as the reverse primer (5' TGCGATCATACCAGCACTAA 3').

We had amplified the *trn*L intron, *trn*L-*trn*F spacer, *mat*K, and *rps*16 intron mostly as a single fragment, but in some cases as two shorter fragments (see Fig. 1 for relative positions of the primers, approximate lengths of the fragments, and references to all primers used). We had amplified the remaining regions as single fragments. Most DNA was extracted, amplified, and sequenced using the ‚regular procedure' (Box 1). For DNA extracts that would not amplify using the ‚regular procedure', a nested PCR approach or a ‚replicate PCR procedure' was used (Box 2). In a few cases, DNA was extracted anew using a ‚replicate DNA extraction procedure' (Box 2). To validate the identity of the obtained sequence, we checked it against nucleotide sequences in GenBank through BLAST searches, and against our own unpublished sequences.

For the *rps*16 intron, which is >800 bp long and one of the longer plastic loci, amplification attempts had been made for 611 accessions of varying age (Fig. 2). As this material is sufficient to perform a formal statistical analysis of the relationship between sequencing success and the age of material, we selected *rps*16 as a conservative measure of DNA quality in this short communication. We used binary sequencing success q of the plastid *rps*16 intron as the response variable in a logistic regression (generalised linear models with logit link function and binomial errors; Venables and Ripley [2002]). The age t of the material (i.e. year

Box 1

The regular procedure
DNA extraction: We crushed 10 to 30 mg of leaf tissue in a 2 mL plastic tube with two tungsten carbide beads for 2 × 1 minute at 30 Hz on a mixer mill (MM301, Retsch GmbH and Co., Haan, Germany). We extracted total DNA from the crushed samples using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) or the E.Z.N.A™ SP Plant DNA Mini Kit (Omega Bio-tek, Inc., Norcross, GA, USA) according to the manufacturers' manual.

PCR amplification: We amplified DNA in 25 µL reactions using the AmpliTaq DNA polymerase buffer II kit (Applied Biosystems, Foster City, California, USA) and 0.2 mM of each dNTP, 0.04% bovine serum albumin (BSA), 0.01 mM tetramethylammonium chloride (TMACl), 0.4 µM of each primer, and 2 µL unquantified genomic DNA. Amplifications were performed in a GeneAmp PCR System 9700 (Applied Biosystems). We performed all PCR amplifications under the following cycling conditions (annealing temperature adjusted according to primer length and GC-content): 95°C for 10', 31 cycles of 95°C for 30", 55-60°C for 30", 72°C for 1', followed by 72°C for 10' and a final hold at 10°C. AmpliTaqGold® DNA Polymerase (Applied Biosystems) was used for amplifying DNA obtained from old herbarium specimens or DNA extracts of reduced quality, whereas AmpliTaq® DNA Polymerase (Applied Biosystems) was used for all high-quality DNA extracts.

PCR purification and sequencing: PCR products were purified using 2 µL 10 times diluted ExoSAP-IT (USB Corporation, Cleveland, Ohio, USA) to 8 µL PCR product, incubated at 37°C for 45 minutes followed by 15 minutes at 80°C. Prepared amplicons for sequencing contained: 9 µL 0-30x diluted purified PCR product (depending on product strength) and 1 µL of 10 µM primer (the same primers as used in the PCR). Cycle sequencing was performed by the ABI laboratory, Department of Biology, University of Oslo. The ABI BigDye Terminator sequencing buffer and v3.1 Cycle Sequencing kit (Applied Biosystems) was used for the cycle sequencing reaction, and sequences were processed on an ABI 3730 DNA analyser (Applied Biosystems).

Box 2

The replicate DNA extraction procedure: We extracted DNA as described in Box 1, but in 2–4 replicate tubes that each included smaller amounts (< 10 mg) of leaf tissue. We performed the DNA elution twice in the same tube using the first eluate in the second elution step. Finally, we pooled DNA extracts from replicate tubes prior to use.

Nested PCR: In this procedure, a second set of amplification cycles are performed using a pair of 'nested' primers sited within the DNA sequence defined by the original primers (Barbara and Garson, 1993). We performed the pre-nested PCR (i.e. the first set of amplification cycles) as described in the regular procedure (Box 1), but with only 25 amplification cycles. As template for the nested PCR (i.e. the second set of amplification cycles), we used a dsH20-diluted (100×) product from the pre-nested PCR, and otherwise identical conditions as described in the regular procedure (Box 1). Optimizations to improve sequence quality included: **1** adjusting the number of amplification cycles in the two separate runs; **2** testing various dilutions (10×–1000×) of the PCR product used as template for the second run.

The replicate PCR procedure: We added template DNA to multiple identical PCR reactions (8–16 tubes) and performed the PCR amplification using the same PCR mix and cycling conditions as described in Box 1, but with 34 cycles. For purification of the PCR products, we added five times the PCR volume of PBI-buffer from the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) to each replicated PCR product before applying all to the same QIAquick DNA-binding column (Qiagen). For the remaining of the procedure, the columns were treated as described in the manufacturer's manual.

of sequencing minus collection year) was used as predictor in this model. The significance of the logistic model was evaluated by comparison with a null model by which only the intercept was modeled, by use of an *F*-test. Modeling results were visualized graphically by showing back-transformed predicted values for sequencing success as a function of age. A 95 % confidence interval for sequencing success as function of age was obtained by inserting $\beta_0 \pm 1.96\text{SE}$ and $\beta_1 \pm 1.96\text{SE}$ into the expression for back-transformed predicted values for sequencing success q, as given by the model:

$$q(t) = \frac{e^{\beta_1 t + \beta_0}}{1 + e^{\beta_1 t + \beta_0}}$$

All calculations were carried out using R version 2.11.1 (R Development Core Team, 2010).

Results and Discussion

A range of mini-prep kits for accomplishing the tissue-to-sequence process, without having to deal with toxic reagents, has become available at a continuously reduced price. However, because of degradation of DNA over time, it is presumed that more comprehensive and laborious techniques, which often include toxic reagents (e.g., Cota-Sanchez et al. 2006), are required to obtain quality DNA from the older material (Fulton and Stiller 2012).

We obtained plastid and nuclear DNA sequences from herbarium specimens up to 168 and 163 years old, respectively (Supplementary Table 1) using regular procedures for DNA extraction and PCR amplification (Box 1). In fact, most specimens collected between 1826 and 1927 did amplify (see Supplementary Table 1), and at least one genetic marker was successfully sequenced for about 90 % of all 651 extracted herbarium specimens.

Of the 611 specimens subjected to *rps*16 sequencing, sequences were obtained for 438 (71.7 %). Although the frequency of specimens in each age class was unevenly distributed, with highest frequency of recently collected material (Fig. 2), a significant relationship between sequencing success and age was found by logistic regression (logit $q = -0.01113 \cdot t + 1.3939$, $p = 2.7 \cdot 10{-}5$, $n = 611$). The model explained 2.43 % of the total deviance. The graph of back-transformed predictions from the model (Fig. 3) shows that the expected sequencing success decreases from c. 80 % for recently collected material to c. 60 % at an age of 100 years. Beyond 100 years, the model indicates a slightly accelerating decrease, but the amount of available old material is insufficient to tell if this is a real trend. Although the model indicates a nearly linear relationship between sequencing success and age, a larger number of old specimens is needed to infer the shape of the relationship, for example if it is close to linear, as indicated (Fig. 3), or logarithmic (i.e., that a constant fraction of successful sequencing trials remains after each doubling of the age of the material).

Both the statistical analysis of results for the rps16 intron and inspection of data for the other markers (Supplementary Table 1) indicate that the upper age limit for herbarium material, from which DNA can be successfully sequenced using regular methods, has not been reached. This is exemplified by the three samples next to the oldest, all of which produced sequences for the DNA regions we attempted to amplify (Supplementary Table 1). Thus, although the negative relationship between age of the material and DNA quality is beyond doubt, other factors are also likely to affect the quality of the DNA. This is evident from the difference in DNA quality and amplification success between equally old accessions of the same species (Supplementary Table 1): the two accessions of *Eriophyton rhomboideum* from 1879 and the two accessions of *Lamium macrodon* from 1902). Already in 1985, Rogers and Bendich wrote „…the extent of DNA degradation for the herbarium specimens appeared to be related to the condition of the leaf rather than the year in which it was dried". Taylor and Swann concluded in 1994 that „...in general, old, air-dried material that has neither been treated with chemical preservatives nor with high heat has the best chance of yielding useful DNA". Our results corroborate their conclusion; our attempts to extract DNA from chemically treated or poisoned specimens never yielded amplicons. It seems that massive parallel sequencing techniques have had more success with such materials (Weiss et al. 2016, Gutaker et al. 2017). More research is needed, however, before we have a full understanding of the conditions other than age and toxic chemicals that affect DNA quality. Such knowledge is important to guide us how to best preserve our valuable collections for the future.

The nested PCR procedure proved successful for obtaining amplicons from degraded DNA. However, optimization was often required to obtain acceptable sequences (Box 2). Moreover, nested PCR is highly prone to contamination, and some of the sequences we obtained were contaminated by modern DNA sources. DNA degradation leads to more fragmented DNA and fewer copies of the entire target sequence for the PCR to work on (Soltis and Soltis, 1993). This, in turn, increases the likelihood of amplifying contaminants. The reason for this is that amplification of damaged or modified DNA is less efficient than amplification of intact template, and that intact DNA always will be amplified preferentially (Pääbo 1989). Although contamination is usually easy to detect by stronger than expected PCR bands as well as the ‚wrong' sequence, it is preferable for obvious reasons to avoid amplification of contaminants altogether. Use of taxon-specific primers and amplification of shorter fragments reduces the risk of contamination.

The ‚replicate PCR procedure' (Box 2) enabled acquisition of DNA sequence data from DNA templates previously discarded as unsuitable for PCR-based methods. Often, only a barely visible band was obtained in one or more of the 8–16 PCR reactions, and in most cases, pooling of near invisible PCR bands resulted

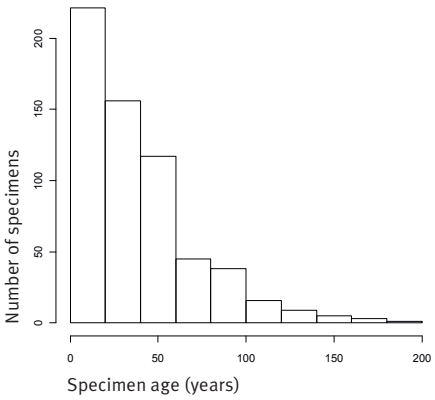

Fig. 2. Histogram of number of specimens subjected to *rps*16 sequencing as a function of the age of the material.

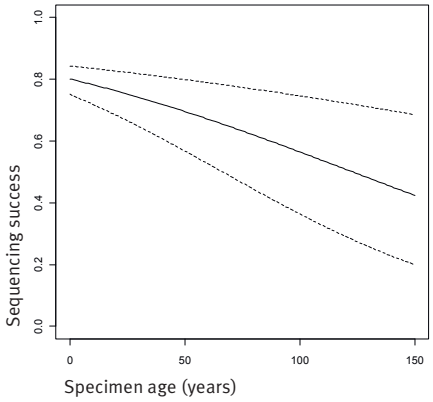

Fig. 3. Predicted relationship between sequencing success (y-axis) and age of material (x-axis), as given by the logistic regression model logit (Sequencing success)= -0.01113·Age + 1.3939 ($p = 2.7 \cdot 10^{-5}$, $n = 611$). Broken lines indicate the 95 % confidence interval for sequencing success.

in excellent sequences. That bands occur in only few of the replicated reactions, suggests that PCR is a somewhat random process that by chance manages to pick up rare fragments. The larger the number of reactions in the replicate procedure, the more likely this is to happen. We have so far never experienced contamination using this method. Increasing the relative volume of template DNA in the PCR reaction never resulted in amplification, possibly due to inhibitory substances extracted along with the old DNA, as previously demonstrated by e.g., Savolainen et al. (1995).

Performing replicate DNA extractions (using smaller volumes of tissue in each tube; Box 2) from the same voucher, with subsequent pooling of the extracts, seems to further increase the chances to obtain good sequences from old herbarium material. In order to minimize destruction of valuable old herbarium specimens, we recommend that this procedure is applied from the beginning in certain cases, instead of extracting larger amount of material in single tubes.

Although neither of the ‚replicate procedures' described in Box 2 would be cost or time efficient for many samples, they may prove useful for complementing datasets with the few difficult-to-amplify templates. Moreover, time and costs can be saved by escaping the need to establish alternative methods that may require additional equipment or chemicals, which is indeed the case still for many labs in the world.

The data used herein were generated as part of a molecular systematic project of the subfamily Lamioideae (Bendiksby et al. 2011a, b, c, 2014; Salmaki et al. 2013, 2015; Scheen et al. 2010)

and were not produced specifically for testing the maximum age of herbarium material that can be sequenced using regular procedures. Thus, the upper limit for fragment length and specimen age is likely to be higher than what is reported herein.

We have reasons to believe that our own studies (e.g., Scheen et al. 2010) are not the only ones in which taxa have had to be omitted due to unsuccessful amplification of DNA extracted from archived specimens. Accordingly, we believe that our modifications to the regular procedure, which significantly increased our ability to obtain DNA sequences from most of the DNA extracts omitted by Scheen et al. (2010) and additional old herbarium specimens (e.g., Bendiksby et al. 2011a, b, c, 2014; Salmaki et al. 2013, 2015), may be of interest to other molecular systematists still using PCR-based methodologies.

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Author contributions statement

Mika Bendiksby designed the study concept and drafted the article. Liseth Thorbek and Mika Bendiksby did the molecular work. Rune Halvorsen did the statistical analyses. Rune Halvorsen and Mika Bendiksby interpreted the results and finalized the manuscript. Charlotte Bjorå was involved in early discussions, commented on and improved the manuscript. All authors approve of the published version.

Conflict of interest statement

The submitted work was not carried out in the presence of any personal, professional, or financial relationships that could potentially be construed as a conflict of interest.

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