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APPLICATION ARTICLE

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Ancient DNA extraction methods for herbarium specimens: When is it worth the effort?

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Abstract

Premise: Herbaria harbor a tremendous number of plant specimens that are rarely used for molecular systematic studies, largely due to the difficulty in extracting sufficient amounts of high-quality DNA from the preserved plant material.

Methods: We compared the standard Qiagen DNeasy Plant Mini Kit and a specific protocol for extracting ancient DNA (aDNA) (the *N*-phenacylthiazolium bromide and dithiothreitol [PTB–DTT] extraction method) from two different plant genera (*Xanthium* and *Salix*). The included herbarium materials covered about two centuries of plant collections. To analyze the success of DNA extraction using each method, a subset of samples was subjected to a standard library preparation as well as target-enrichment approaches.

Results: The PTB–DTT method produced a higher DNA yield of better quality than the Qiagen kit; however, extracts from the Qiagen kit over a certain DNA yield and quality threshold produced comparable sequencing results. The sequencing resulted in high proportions of endogenous reads. We were able to successfully sequence 200-year-old samples.

Discussion: This method comparison revealed that, for younger specimens, DNA extraction using a standard kit might be sufficient. For old and precious herbarium specimens, aDNA extraction methods are better suited to meet the requirements for next-generation sequencing.

KEYWORDS

archival DNA, genome skimming, herbarium genomics, *Salix*, target enrichment, *Xanthium*

Molecular biodiversity research and phylogenomic studies rely on comprehensive sampling; however, the required material is often not available, either because of extinct species or because the species occur in very remote areas. To overcome the problems of insufficient sampling, herbarium specimens could be used (Staats et al., 2011, 2013). Herbaria harbor a massive number of specimens collected over several centuries, and are therefore considered treasure troves for biodiversity research (Bebber et al., 2010; Xu et al., 2015; Besnard et al., 2018; Funk, 2018; Alsos et al., 2020). It is estimated that around 70,000 new species are already housed in herbaria, "waiting to be described" (Bebber et al., 2010).

Although herbarium vouchers are a valuable source of information, using them for molecular studies remains challenging (Staats et al., 2012; Xu et al., 2015). DNA from herbarium specimens is usually highly degraded and fragmented, making its extraction from old tissues particularly difficult. The generally limited success of DNA extraction and the challenges associated with the PCR amplification of highly degraded DNA means that researchers often avoid including historical specimens (Xu et al., 2015). While Sanger sequencing usually requires long and intact DNA fragments, recent developments in sequencing techniques have enabled researchers to include fragmented DNA in their approaches (Bakker et al., 2016; Alsos et al., 2020). Nevertheless, a certain level of DNA quality and quantity is necessary to include

historical material in studies using next-generation sequencing (NGS) methods.

For most phylogenomic studies, DNA is usually extracted from fresh or silica-dried plant material using a commercial DNA extraction kit. Historical samples require more advanced methods, with special regard paid to their shorter fragments and potential contamination (Gutaker and Burbano, 2017). Moreover, extracting DNA from plant cells is intrinsically more complicated than extraction from animal cells, especially for historical samples. Weiß et al. (2016) found that plant DNA in herbaria showed a six-fold higher fragmentation rate than animal DNA preserved in bones. A high number of plant secondary compounds, including polyphenolics and polysaccharides, can covalently bind to DNA or coprecipitate with it, inhibiting PCR even in non-degraded DNA samples. This further complicates the usage of DNA from plant herbarium tissues (Kistler, 2012; Alsos et al., 2020). Additionally, the quality and quantity of DNA found in herbarium specimens depend on the conditions to which the specimens were exposed during collection and storage, and are, in general, lower than for freshly collected, silica-dried, or frozen plant materials (Staats et al., 2011; Drábková, 2014; Lang et al., 2019).

The first trials to extract ancient DNA (aDNA) and/or archival DNA (also known as historical DNA [hDNA]; Raxworthy and Smith, 2021) from plant remains began in the early 1990s (e.g., Soltis et al., 1992; Brown et al., 1994), but their success was later questioned (Austin et al., 1997). Studies of herbarium material aiming to sequence single markers (e.g., ITS) used the standard cetyltrimethylammonium bromide (CTAB) protocol for DNA extraction (Albach and Chase, 2001), or a modified version of it (Kistler, 2012; Clayton and Roberts, 2014; Höpke et al., 2019; de Castro et al., 2021). In other studies, commercial kits were used with a few adaptations, such as increasing the incubation times (Clayton and Roberts, 2014; Dwivedi et al., 2018; Villaverde et al., 2018; Höpke et al., 2019). Finally, more specific protocols for aDNA extraction were developed, with optimizations to obtain shorter fragments and to increase the proportion of endogenous DNA (Kistler, 2012; Drábková, 2014; Gutaker and Burbano, 2017; Shepherd, 2017). Since then, scientists have increasingly included historical plant material in phylogenomic studies (Hart et al., 2016; Zedane et al., 2016; Villaverde et al., 2018).

The aDNA-specific protocols are generally more expensive, more time consuming, and require specific facilities and contamination-avoidance protocols that might not always be available in systematic botany laboratories. Gutaker et al. (2017) adapted a protocol originally designed to extract DNA from hominin fossils (Dabney et al., 2013) for use with old herbarium specimens. The main modifications were the inclusion of *N*-phenacylthiazolium bromide (PTB) and dithiothreitol (DTT) in the lysis buffer. PTB facilitates the release of small DNA fragments trapped in sugar-derived condensation products (Poinar et al., 1998), whereas DTT reduces disulfide bonds, making thiolated DNA from the cross-linked complexes available (Gill et al., 1985). This method (hereafter referred to as the PTB–DTT extraction method) outperformed the CTAB extraction in terms of the proportion of small fragment and endogenous DNA obtained (Gutaker et al., 2017).

For systematists (systematic botanists), extraction kits still represent the easiest and most convenient solution for DNA extraction. Although a few recent studies focused on comparing the efficiency of CTAB-based extraction protocols with commercial kits (Höpke et al., 2019), or comparing CTAB extractions with protocols specific for aDNA (Gutaker et al., 2017), no studies yet have investigated the circumstances in which aDNA methods would be preferred to commercial kits when extracting hDNA from old and damaged herbarium materials.

In the study presented here, we investigate when it would be better to invest more time and resources into extracting DNA from herbarium specimens using a specific aDNA protocol (PTB–DTT), and under which circumstances a standard kit would be sufficient. We measure the yield and quality of the DNA obtained from herbarium materials of different ages and conditions using the PTB–DTT approach and the standard Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Additionally, we assess the extraction success by subjecting the resulting DNA to a standard NGS library preparation (i.e., double-stranded library preparation for Illumina [San Diego, California, USA] sequencing) and target enrichment approaches using commercially available kits. To incorporate the taxonomic effect on extraction performance, we used specimens from a total of seven species from two phylogenetically very distantly related plant genera.

METHODS

Plant materials

We used herbarium materials from two plant genera, *Salix* L. (Salicaceae) and *Xanthium* L. (Asteraceae). For the genus *Salix*, we included four samples from each of three species: *S. caprea* L., a diploid tree or large shrub that is distributed across central Europe; *S. myrsinifolia* Salisb., a widely distributed hexaploid tree; and *S. breviserrata* Flod., an alpine diploid dwarf shrub. The herbarium samples were obtained from the Herbarium of the University of Göttingen (GOET; herbarium acronyms per Index Herbariorum [Thiers, 2022]) and covered about two centuries. The oldest herbarium sheet was from 1820, while the youngest was from 2015.

For *Xanthium*, we included samples from the two sections of the genus: section *Xanthium* (plants with unarmed stems) and section *Acanthoxanthium* DC. (plants with spiny stems). The specimens were obtained from GOET, the Herbarium of the Botanic Garden and Botanical Museum Berlin-Dahlem (B), and the Herbarium of the

TABLE 1 Information on the herbarium specimens of *Xanthium* and *Salix* used in this study, including the year of collection, DNA concentration, and the resulting absorbance ratio values for the Qiagen DNeasy Plant Mini Kit and the PTB–DTT extractions. Successful PCR amplifications are indicated by the symbol *x*, PCR failures by °. Species assignment in *Xanthium* follows Tomasello (2018).

Herbarium voucher	Species	Lab ID	Year of collection	Qiagen DNeasy Plant Mini Kit				PTB–DTT			
				Conc. (ng/μL)	A_{260} : A_{280}	A_{260} : A_{230}	PCR test	Conc. (ng/μL)	A_{260} : A_{280}	A_{260} : A_{230}	PCR test
GOET0590898	*S. breviserrata* Flod.	brevi1900	1900	0.33	1.54	0.63	°	15.9	2.06	2.16	°
GOET0590900	*S. breviserrata*	brevi1981	1981	0.80	1.66	0.47	x	8.0	1.97	1.87	x
GOET0590901	*S. breviserrata*	brevi2000	2000	9.21	1.69	1.37	x	44.9	2.11	2.16	x
GOET0590899	*S. breviserrata*	brevi2015	2015	5.08	1.57	1.22	x	30.1	2.12	2.29	x
GOET0590894	*S. caprea* L.	caprea1851	1851	1.34	1.19	0.44	°	5.2	1.85	2.96	x
GOET0590895	*S. caprea*	caprea1904	1904	34.10	1.77	1.70	x	11.2	2.30	2.30	x
GOET0590896	*S. caprea*	caprea1981	1981	3.21	1.36	0.59	x	54.0	2.29	2.29	°
GOET0590897	*S. caprea*	caprea2014	2014	37.60	1.79	2.18	x	52.0	2.34	2.34	x
GOET0590890	*S. myrsinifolia* Salisb.	myrsi1820	1820	0.07	1.46	0.75	°	17.3	2.07	2.33	°
GOET0590892	*S. myrsinifolia*	myrsi1873	1873	0.20	1.29	0.50	°	19.3	2.07	2.27	°
GOET0590891	*S. myrsinifolia*	myrsi1895	1895	0.55	1.59	1.70	°	30.5	2.09	2.32	°
GOET0590893	*S. myrsinifolia*	myrsi2014	2014	6.20	1.54	0.64	x	18.5	2.08	2.15	x
GOET042893	*X. chinense* Mill.	X129	1882	45.40	1.70	1.58	x	<60	1.81	2.30	°
M-0158776	*X. chinense*	X12	1965	15.10	1.83	1.80	x	56.0	1.81	1.89	x
GOET042888	*X. orientale* L.	X133	1830	19.30	1.60	1.08	°	48.7	1.80	2.16	°
GOET042966	*X. orientale*	X136	1851	7.04	1.79	1.98	°	31.5	1.88	2.33	°
GOET042625	*X. orientale*	X125	1852	6.28	1.65	1.10	°	40.4	1.85	2.20	x
GOET042646	*X. orientale*	X127	1853	4.80	1.70	1.18	°	26.8	1.91	2.24	°
GOET042644	*X. orientale*	X131	1872	4.02	1.65	1.02	°	14.7	1.90	2.18	°
GOET042880	*X. orientale*	X132	1874	19.30	1.67	0.90	°	42.7	1.85	2.19	x
GOET042652	*X. orientale*	X128	1882	6.46	1.75	1.24	°	15.8	1.86	2.04	°
GOET042963	*X. orientale*	X135	1896	6.32	1.61	1.14	x	53.0	1.98	2.36	x
GOET042645	*X. orientale*	X126	1907	6.76	1.58	0.87	°	35.5	1.82	2.06	°
M-0158769	*X. orientale*	X3	1965	7.94	1.71	1.15	°	44.8	1.79	2.28	x
GOET042659	*X. orientale*	X120	1973	13.60	1.75	0.63	x	23.3	1.77	2.12	x
GOET042886	*X. orientale*	X122	1973	24.50	1.8	1.53	x	42.6	1.81	2.38	x
B 10 0467877	*X. orientale*	X29	1983	51.00	1.47	1.34	°	55.0	1.79	1.95	x
B 10 0467884	*X. orientale*	X31	1984	47.00	1.92	1.10	x	60.0	1.82	2.18	x
GOET043090	*X. spinosum* L.	X137	1840	16.50	1.69	1.43	°	17.8	1.74	1.88	x
GOET043095	*X. spinosum*	X130	1870	11.60	1.52	0.85	°	48.6	1.79	2.05	x
GOET042990	*X. spinosum*	X119	1903	6.73	1.56	0.84	°	21.9	1.78	2.06	x
GOET043085	*X. spinosum*	X124	1924	31.80	1.72	1.54	°	37.0	1.81	2.34	x
GOET042994	*X. spinosum*	X123	1934	35.10	1.73	1.45	°	53.0	1.79	2.28	x
B 10 0467880	*X. spinosum*	X26	1940	10.90	1.82	0.84	°	29.6	1.82	1.94	x
GOET042660	*X. spinosum*	X121	1957	19.40	1.57	0.96	°	49.4	1.81	2.39	x
M-0158771	*X. spinosum*	X6	1963	22.30	1.72	1.07	x	51.0	1.78	2.18	x
GOET043118	*X. strumarium* L.	X134	1821	5.98	1.78	1.46	x	24.4	1.89	2.67	x

Bavarian Natural History Collections (M), with the oldest being collected in 1821 and the youngest in 1984. In total, we used 25 *Xanthium* accessions. For details of all samples used in this study, see Table 1.

DNA extraction

For each sample, 10 mg of leaf material was removed from the herbarium sheet, transferred into an Eppendorf tube, then pulverized using a TissueLyser II (Qiagen). Both extraction methods were applied to each sample. The PTB–DTT extractions were performed as described by Dabney et al. (2013), following the modifications applied by Gutaker et al. (2017). The DNA of all samples was also extracted using a Qiagen DNeasy Plant Mini Kit, according to the manufacturer's instructions and with the following modifications (Wagner et al., 2018): (1) the lysis incubation and the incubation on ice after adding the P3 buffer were each prolonged to 30 min (instead of 10 and 5 min, respectively); (2) during the DNA elution, 50 µL of AE buffer (instead of 100 µL) was added to the column and incubated for 30 min (instead of 5 min) before centrifugation. The elution step was repeated, resulting in 100 µL of extracted DNA.

All extractions were performed under contamination-avoidance measures typical for working with aDNA. Surfaces and consumables were sterilized with DNA AWAY (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and pipettes were UV-treated using a nUVaClean UV Pipette Carousel (MTC Bio, Sayreville, New Jersey, USA). Extractions were carried out under a laminar flow hood wearing gloves, a mask, and a full-body laboratory suit.

DNA yield and quality measurements

Because the same amount (10 mg) of herbarium material was employed in each extraction, we used concentrations as a measure of DNA yield. The concentrations were measured on a Qubit 3 Fluorometer (Thermo Fisher Scientific) using the Qubit dsDNA HS Assay kit (Thermo Fisher Scientific). To measure the A_{260} : A_{280} and A_{260} : A_{230} absorbance ratios, we used a NanoDrop 2000 (Thermo Fisher Scientific).

Additionally, we ran the samples on electrophoresis gels to visually check the success of the extractions and determine the approximate fragment lengths. We mixed 5 µL of DNA extract with 1 µL of Roti-Load DNAstain 3 (Carl Roth, Karlsruhe, Germany), and loaded it in a 2% agarose gel. Electrophoreses were run for 40 min at 100 V.

Statistics

To test for a correlation between the age of the herbarium specimen and the DNA yield obtained, we performed Pearson correlation tests (Pearson, 1900), treating samples from the two genera, as well as the two extraction methods, separately. An analysis of covariance (ANCOVA) was performed to test the effect of the extraction method (Qiagen kit vs. PTB–DTT) and the taxonomy on the DNA yield (DNA concentration) and quality (A_{260} : A_{280} and A_{260} : A_{230} absorbance ratios), treating the voucher age as a covariate. We tested ANCOVA assumptions for normality and homoscedasticity using Levene's test (Levene, 1960). All statistical analyses, as well as the generation of the scatterplots and boxplots, were performed in R (R Core Team, 2018).

PCR test

As an additional quality check, the extracted DNA was used to amplify the plant plastid locus *trnL-trnF* with the primers *e* and *f* (Taberlet et al., 1991). For each sample, 1 µL of the sample was mixed with 12.5 µL of Roti-Pol TaqS Master Mix (Carl Roth), 1 µL each of forward and reverse primers (5 pmol/µL), and 9 µL of sterile distilled water, for a final volume of 25 µL. We used a touchdown protocol for amplification: denaturation at 94°C for 2 min; 10 cycles each starting with 20 s at 94°C, 20 s at 63°C with a drop of 1°C per cycle, and 30 s at 72°C; 25 cycles of 20 s at 94°C, 20 s at 52°C, and 30 s at 72°C; with a final extension of 72°C for 5 min. To check the amplification success, 1 µL of the PCR product was mixed with 4 µL of ddH_2O and 1 µL of Roti-Load DNAstain 3 (Carl Roth), loaded onto a 2% agarose gel, and run for 40 min at 100 V.

Library preparation and sequencing

To estimate the amount of endogenous DNA (i.e., percentage of reads mapping to a reference) and to analyze whether the extracts were usable for NGS, we sequenced a subset of 12 samples (six *Salix* and six *Xanthium* samples from both extraction methods) using an Illumina system. The libraries were prepared using either the NEBNext Ultra II DNA Library Prep Kit for Illumina (for old herbarium specimens in which DNA fragment length did not exceed 500 bp) or the NEBNext Ultra II FS DNA Library Prep Kit for Illumina (for more recent specimens) (New England BioLabs, Ipswich, Massachusetts, USA). In both cases, we followed the manufacturer's instructions, with a single modification in the purification step following the adapter ligation: we used 1.5 volumes of HighPrep beads (MagBio Genomics, Gaithersburg, Maryland, USA) instead of 0.8 volumes, to minimize the loss of ultra-short fragments. The samples were PCR amplified for 14 cycles, and sample-specific dual indices (NEBNext Multiplex Oligos for Illumina, New England BioLabs) were attached to the fragments.

For *Salix*, the reads could be mapped to an available reference genome. No *Xanthium* genome is available; therefore, we used the target regions of the bait kit as a "pseudoreference" for read mapping, enabling an estimation of the proportion of endogenous DNA. In this way, we could also investigate whether the libraries were suitable for a hybrid capture reaction. Standard kits have 120-bp

baits and might not efficiently hybridize the ultra-short fragments of very old herbarium specimens; thus, the *Xanthium* samples were subjected to a hybrid capture reaction using the commercially available myBaits COS Compositae 1Kv1 kit (Daicel Arbor Biosciences, Ann Arbor, Michigan, USA). Six indexed samples were pooled in equal quantities, dehydrated in a Concentrator Plus (Eppendorf, Hamburg, Germany), and diluted in 7 μL of ddH_2O. The pool was enriched using the bait kit, following the manufacturer's protocol. Hybridization took place for 20 h at 65°C. The enriched products were PCR amplified for 14 cycles using a 2X KAPA HiFi HotStart Mix (Roche, Basel, Switzerland) and the P7 and P5 adapters as primers. The concentrations were measured on a Qubit 3 Fluorometer (Thermo Fisher Scientific), and the fragment length distribution was checked with a QIAxcel (Qiagen). The *Salix* libraries presented adapter-dimer peaks at around 125 bp and were therefore treated with a BluePippin (Sage Science, Beverly, Massachusetts, USA) to select fragments between 140 and 600 bp in length, using a 2% cartridge and an internal standard. Finally, the samples (six *Salix* libraries and the *Xanthium* hybrid capture pool) were pooled equimolarly and paired-end sequenced on an Illumina MiSeq System at the NGS Integrative Genomics Core Unit (University of Göttingen), using a 2 × 150 bp (300 cycles) v2 kit.

Read quality check, mapping, and plastome reconstruction

The resulting reads were quality checked using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The sequence adapters were removed, and the reads were quality-trimmed using Trimmomatic version 0.32 (Bolger et al., 2014), with default settings. To analyze the percentage of endogenous reads, the reads of the six *Salix* samples were mapped to the published *S. purpurea* L. reference genome (female clone 94006; *Salix purpurea* version 5.1; U.S. Department of Energy Joint Genome Institute [DOE-JGI]; http://phytozome.jgi.doe.gov/). The reads of the six *Xanthium* samples were mapped to a reference consisting of the concatenated target exon sequences, each separated by stretches of 800 Ns. Mapping was performed using the BWA-MEM algorithm of the Burrows–Wheeler Aligner version 0.7.12 (Li and Durbin, 2009), with default settings. The quality-filtered reads were also used to reconstruct the plastome for each sample, for which the reads were subjected to a reference-based assembly using Geneious version R11 2020.2.4 (http://www.geneious.com; Kearse et al., 2012), as described by Ripma et al. (2014). As references, we used the plastomes available in GenBank (National Center for Biotechnology Information) for each species, i.e., *S. breviserrata* (MW435421), *S. caprea* (MW435424), *S. myrsinifolia* (MW435439), and *X. sibiricum* Patrin ex Widder (MH473582).

RESULTS

DNA yield

In total, the DNA of 37 samples was extracted using both the PTB–DTT method and the standard Qiagen DNeasy Plant Mini Kit. The results of the gel electrophoreses for all extracts are shown in Appendix S1. The observed DNA concentrations were significantly higher in the PTB–DTT extractions (mean = 34.87 ng/μL) than those extracted using the Qiagen kit (mean = 14.70 ng/μL) when considering the complete data set (paired Student's *t*-test, $P < 0.01$; Figure 1A). The DNA concentrations obtained were slightly negatively correlated with the age of the herbarium specimen (Pearson's $r = 0.34$ [$P = 0.042$] and $r = 0.30$ [$P = 0.071$] for the PTB–DTT and the Qiagen kit, respectively; Figure 1B). The taxon effect (*Salix* vs. *Xanthium*) was also significant ($P = 0.0096$), indicating that the concentrations of *Xanthium* DNA extracts (mean = 28.57 ng/μL) were significantly higher than those of *Salix* (mean = 16.90 ng/μL).

When treating the two genera separately, the results were similar to those presented above. In both cases, the PTB–DTT extractions performed better than the Qiagen kit ($P < 0.001$ and $P = 0.007$ in *Xanthium* and *Salix*, respectively; see Appendix S2). The taxonomic effect (i.e., differences among the different species of *Salix* or sections of *Xanthium*) was not significant in *Salix* ($P = 0.184$) or in *Xanthium* ($P = 0.909$). As for the complete data set, the concentrations were slightly negatively correlated with the age of the specimens, both in *Xanthium* ($r = 0.43$ [$P = 0.031$] and $r = 0.47$ [$P = 0.018$] in the PTB–DTT and the Qiagen kit extractions, respectively; Appendix S3A) and in *Salix* ($r = 0.56$ [$P = 0.060$] and $r = 0.31$ [$P = 0.33$]; Appendix S3B); however, this correlation was not significant in *Salix*.

DNA quality

A high-quality DNA extract shows an $A_{260} : A_{280}$ ratio of 1.8 and an $A_{260} : A_{230}$ ratio above 2.0. Our results revealed that the DNA quality was higher for the PTB–DTT extractions than those obtained using the Qiagen kit; the $A_{260} : A_{280}$ ratios were significantly higher ($P < 0.001$) for the PTB–DTT extracts (mean = 1.92) than the Qiagen kit (mean = 1.64) (Figure 2A). The results of the $A_{260} : A_{230}$ ratios could not be statistically compared because the groups showed a significant heterogeneity in their variances (Levene's test, $P < 0.001$) (Figure 2B).

PCR test

The success of the amplification of the plastid *trnL-trnF* spacer was assessed by the presence of a visible band at a length of approximately 430 bp on the agarose gel. The amplification was successful for 25 of the 37 samples

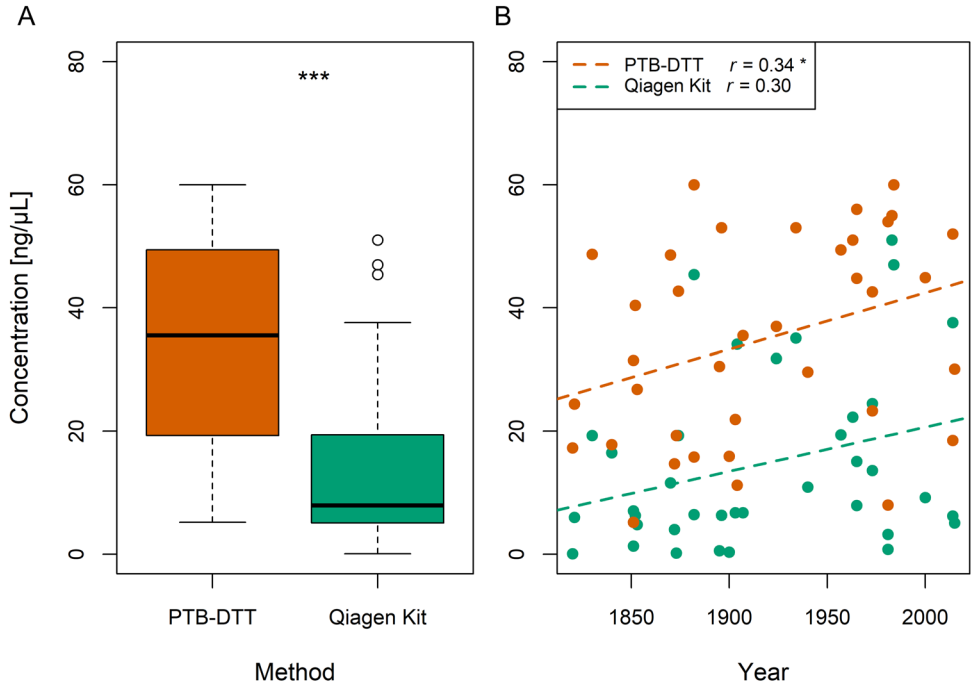

FIGURE 1 Comparison of the DNA concentrations (in ng/µL) obtained in all extracts produced using the two extraction methods. (A) Boxplots of the DNA concentrations of all samples extracted using the PTB–DTT and Qiagen DNeasy Plant Mini Kit protocols. Asterisks represent statistical significance: (***) $P < 0.001$. (B) Scatterplot of the DNA concentrations of all extracted samples against the age of the respective herbarium sheets (year of origin). The lines represent a general linear model for the relationship between the DNA concentration and the year of the herbarium sheet for the PTB–DTT and Qiagen Kit protocols. Value *r* represents the calculated Pearson correlation coefficient. Asterisk represents a statistically significant linear relationship: (*) $P < 0.05$.

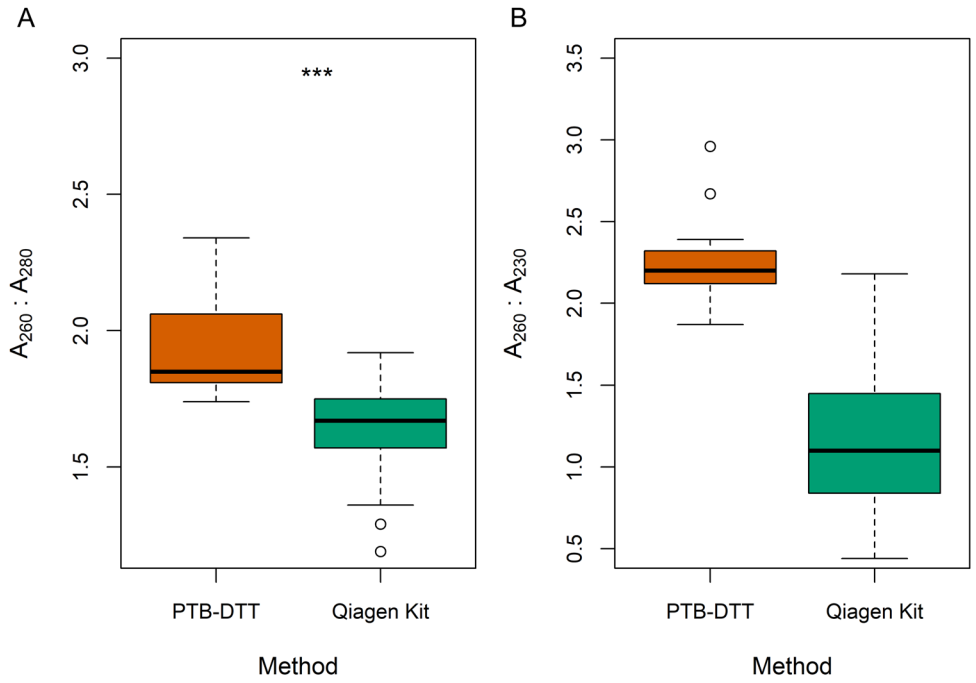

FIGURE 2 Comparison of the DNA quality obtained in all extracts produced using the two extraction methods. (A) $A_{260} : A_{280}$ ratios measured for all samples extracted using the PTB–DTT and Qiagen DNeasy Plant Mini Kit extraction protocols. (B) $A_{260} : A_{230}$ ratios measured for all samples obtained using the two extraction protocols. Asterisks represent statistical significance: (***) $P < 0.001$.

extracted using the PTB–DTT method, and for 15 of the 37 samples extracted with the Qiagen kit. A total of 26 *Xanthium* samples (of 50 amplifications) and 14 *Salix* samples (of 24) were successfully amplified across both extraction methods (see Table 1 for details).

NGS results

The sequencing produced 31,899,780 reads in total. On average, we obtained 2,658,315 reads per sample, ranging from 979,024 reads (*X. spinosum* L., X137 PTB) to 4,254,576 reads (*X. orientale* L., X133 PTB). The number of filtered low-quality reads after trimming differed between the two genera. In *Salix*, the percentage of reads excluded by the quality trimming was 13.5% (9.13–21.45%), whereas in *Xanthium* 1.44% (1.06–1.85%) of reads were removed. The percentage of duplicated reads was 0.82% (0.32–1.80%) in *Salix* and 20.53% (14.98–34.50%) in *Xanthium*. The average number of reads remaining after the quality and duplicate filtering was 2,632,716 for the *Salix* samples and 1,709,997 in *Xanthium*. The average percentage of mapped reads was 85.08% in *Salix* (78.61–89.05%) and 62.6% in *Xanthium* (55.91–69.38%) (Table 2).

The plastome assembly was able to recover 100% of the plastomes of the three *Salix* species, with 5.59–11.76% of filtered reads mapping to the respective reference plastomes. The mean coverage varied between 38 and 104 reads. For both *Xanthium* sections, 0.09–3.13% of filtered reads mapped to the reference and between 31% and 83% of the plastome could be recovered. The mean coverage varied between one and 210 reads. For more details, see Table 2.

DISCUSSION

Comparison of the extraction methods

Extraction methods specifically developed for old archaeobotanical remains outperform standard extraction methods, both in terms of DNA yield and the proportion of small endogenous DNA fragments (Gutaker et al., 2017). In our study, we confirmed that the PTB–DTT methods produced higher yields than a widely used extraction kit. In some cases, such as for old *Salix* herbarium specimens, using the PTB–DTT extraction method was the only means of obtaining sufficient DNA for a library preparation.

The PTB–DTT method also produced a higher quality of DNA extracts (as measured by the absorbance ratios A_{260} : A_{280} and A_{260} : A_{230}) than the Qiagen kit. Our results differ from those of a previous study (Höpke et al., 2019), in which a silica column–based extraction kit produced purer DNA than the CTAB method. The high performance of the PTB–DTT method could be explained by the fact that the DNA precipitation was also performed on a silica column, producing high-quality extracts. Moreover, in our study, the lower quality of the kit extracts could be partially biased, on account of the low absorbance values measured in the extracts of the old herbarium specimens with extremely low DNA concentrations.

The success of the amplification was dependent on the extract quality and concentration. In general, and according to our expectations, relatively young herbarium specimens performed better than the older ones. A higher number of PTB–DTT extractions produced good amplifications (25 samples) than those extracted with the kit (15 samples).

TABLE 2 Results from sequencing the 12 samples selected for the library preparation. Details for sample IDs are provided in Table 1.

								Genome/targeted regions		Plastome	
Sample ID	Species	Total no. of reads	No. of quality-trimmed reads	% quality-trimmed reads	No. of reads without duplicates	% duplicates	No. of paired reads without duplicates	No. of mapped reads	% of mapped reads	No. of mapped (paired) reads	% of mapped reads
X127 PTB	*X. orientale*	1,664,846	1,634,471	1.83	1,389,725	14.98	810,379	898,313	64.64	18,760	2.31
X133 PTB	*X. orientale*	4,254,576	4,209,480	1.06	2,757,444	34.50	2,087,569	1,853,679	67.22	1,905	0.09
X135 PTB	*X. orientale*	3,035,086	3,000,746	1.14	2,535,678	15.50	1,488,551	1,759,861	69.40	8,382	0.56
X119 PTB	*X. spinosum*	1,539,976	1,511,586	1.85	1,261,404	16.56	745,772	743,944	58.98	23,343	3.13
X137 PTB	*X. spinosum*	979,024	964,606	1.48	752,872	21.96	477,935	420,956	55.91	2,145	0.45
X137 QIA	*X. spinosum*	1,970,678	1,945,724	1.27	1,562,864	19.68	963,618	928,844	59.43	28,281	2.93
brevi2000 PTB	*S. breviserrata*	4,207,912	3,558,700	15.43	3,544,188	0.41	1,507,542	2,826,025	79.74	175,488	11.64
brevi2000 QIA	*S. breviserrata*	3,059,196	2,779,898	9.13	2,771,084	0.32	1,302,529	2,496,801	90.10	72,785	5.59
caprea1981 PTB	*S. caprea*	2,808,198	2,402,821	14.44	2,359,571	1.80	1,102,771	2,015,842	85.43	89,037	8.07
caprea2014 PTB	*S. caprea*	3,084,266	2,756,045	10.65	2,743,545	0.46	1,273,101	2,425,974	88.42	119,865	9.42
caprea2014 QIA	*S. caprea*	2,271,790	2,044,809	10.00	2,029,125	0.77	982,885	1,827,017	90.04	88,303	8.98
myrsi1820 PTB	*S. myrsinifolia*	3,024,232	2,375,746	21.45	2,348,788	1.14	1,138,641	1,938,528	82.53	133,866	11.76

Note: PTB = PTB–DTT extraction protocol; QIA = Qiagen DNeasy Plant Mini Kit.

The quality of the extracts (i.e., the purity of the DNA) is particularly important for the success of PCR-based techniques (Drábková et al., 2002; Wales et al., 2014). This was confirmed by the lower success of the PCR amplifications using the kit extractions, especially for the older herbarium specimens. For samples predating 1900, only three and seven PCR reactions produced bands for the kit and the PTB–DTT extracts, respectively. Additionally, the overall concentration of the DNA had an impact on the PCR success, and was generally higher in the PTB–DTT extractions. Moreover, the amplification of the 430-bp PCR product was successful when using the fragmented DNA samples (see Appendix S1) that showed a majority of fragments between 200 bp and 500 bp.

Regarding the two genera, more of the *Xanthium* amplifications were successful than the *Salix*. This is probably because the *Xanthium* extractions generally had a better DNA yield and quality than the *Salix* samples, especially for the old herbarium specimens (see Table 1). Furthermore, willows (*Salix* spp.) are rich in secondary compounds, such as salicylates, tannins, and flavonoids (Palo, 1984; Piątczak et al., 2020), which might unfavorably affect the performance of the DNA extractions and the downstream analyses.

Effect of specimen age on DNA yield and quality

In the present study, we extracted archival DNA from 37 herbarium specimens, with ages spanning 200 years. Our results were similar to those reported by Zeng et al. (2018), in that we found a negative correlation between the age of the specimens and the DNA yield obtained. Older samples generally had a lower yield, especially when using the commercial extraction kit. Our results contrast with those of other studies (Bakker et al., 2016; Höpke et al., 2019), where no correlation was found between the age of the specimens and DNA yield. The reason for this discrepancy might be explained by sampling peculiarities. Höpke et al. (2019) employed herbarium specimens that were no more than 60 years old, while Bakker et al. (2016) used both fresh and herbarium samples, with most of the latter being not more than 60 years old. However, this does not necessarily mean that the DNA yield obtained from a very old sample is always lower than that from recent herbarium specimens; the extent to which the DNA of an old herbarium voucher is degraded depends on other factors for which information is usually scarce (e.g., specimen preparation and conservation conditions). One would expect that plants collected and desiccated in cool and dry environments would yield higher quantities of less-degraded DNA than plants collected under wet and tropical conditions. Although thus far only a few studies have tried to investigate these aspects (e.g., Kates et al., 2021), Bakker et al. (2016) found that, based on read assembly results, the fragmentation effects caused by the age of the sample were more consistent in materials from wet and tropical environments, probably due to the longer and more destructive preparation methods used (e.g., heat, alcohol).

Moreover, the efficiency of the extraction methods in old specimens may differ considerably in different taxonomic groups (Höpke et al., 2019). In our study, we compared specimens from taxa of two systematically very distant genera. The negative effect of age was much more drastic in *Salix* than in *Xanthium* (Appendix S3). When using a standard extraction kit, *Salix* samples older than 100 years could not produce DNA yields high enough to be employed in standard (double-stranded DNA) library preparation methods (DNA concentrations between 0.069 and 1.34 ng/μL were obtained from samples predating 1900; Table 1). On the other hand, the Qiagen kit performed relatively well for *Xanthium* (in terms of DNA yield), even in samples up to 200 years old.

Specimen age, and especially the extent of DNA fragmentation, seems to have a strong effect on the success of PCR amplification. DNA extracts from old specimens have higher proportions of short fragments than those of younger samples (see also Appendix S1). The negative effect of specimen age on PCR success explains the results of our PCR amplification test, in which approximately 41% of specimens (7/17 extracts) from the 19th century extracted using the PTB–DDT protocol were successfully PCR amplified, compared with 90% (18/20 extracts) of samples from the 20th and 21st centuries (Table 1). The PTB–DDT extracts generally had sufficiently high concentrations, but a high concentration alone was not sufficient for a successful PCR amplification. Nevertheless, high proportions of small fragments were not crucial for the performance of NGS, and samples that did not produce PCR bands (e.g., "capr1981" or "myrsi1820") were still able to produce sufficient NGS reads to reconstruct the complete species plastome (see discussion below).

Library preparation for Illumina sequencing

We produced libraries for Illumina sequencing for 12 of the 37 samples included in the study, using PTB–DTT and Qiagen kit extracts. This was done to assess the proportion of endogenous DNA and to test whether the extractions could be successfully used for library preparation. For the *Salix* samples, the libraries were directly sequenced and mapped onto a *Salix* reference genome. For *Xanthium*, the libraries were enriched using a commercially available bait kit, and target regions were subsequently used as "pseudoreferences." This also enabled us to investigate how a commercial kit (noncustomized for archival DNA) performed on libraries obtained from old herbarium vouchers.

Based on our results, we observed a relatively high proportion of low-quality reads in *Salix*. This could be attributed to the high number of short and damaged DNA fragments obtained from extractions using old and

degraded herbarium vouchers; however, the degraded DNA samples showed a majority of fragments in the range of 200–300 bp. In "capr1981" or "myrsi1820," for example, the amplification of the *trnL-trnF* spacer failed, but Illumina sequencing resulted in a sufficient number of reads to reconstruct the complete plastome (see below). When comparing both extraction methods, sequencing the PTB–DTT extracts resulted in a higher number of reads than sequencing the Qiagen kit extracts. Thus, in *Salix*, the DNA concentration had a higher impact on the number of reads than the level of degradation. In *Xanthium*, only a small proportion of reads were filtered out due to low quality. The hybrid-capture reaction probably helped to mitigate this problem by enriching the libraries of DNA fragments capable of binding to the baits (e.g., fragments that were long enough and not degraded).

The number of duplicate reads was relatively high in *Xanthium*. Clonality has been reported as a potential problem when target-enrichment techniques are applied to old and damaged DNA (Ávila-Arcos et al., 2011). This is particularly evident when high numbers of (post-capture) PCR cycles are performed on samples with low proportions of endogenous and/or damaged DNA (such as old herbarium samples). Increasing the amount of starting DNA (Hart et al., 2016) or pooling multiple shorter, independent amplifications of a library (Ávila-Arcos et al., 2011) may help to solve this issue. In general, there are a few factors intrinsic to DNA extracted from old and degraded tissues that influence the efficiency of the in-solution hybrid capture reactions (e.g., low levels of endogenous DNA, very short DNA fragments; Lan and Lindqvist, 2018). A few adaptations to the standard protocol may help to partially overcome these problems, including increasing the amount of starting DNA (Hart et al., 2016) or decreasing the hybridization temperature (Cruz-Dávalos et al., 2017).

In *Salix*, 80–90% of the reads (after quality filtering) mapped to the reference genome, providing evidence of high proportions of endogenous DNA even in old herbarium specimens. For the oldest sample sequenced (*S. myrsinifolia* from 1820), about 82% of the reads mapped to the reference genome. In a similar study, only a few samples achieved such mapping success (Gutaker et al., 2017). Our results confirm that standard double-stranded library preparation (as an alternative to the more expensive single-stranded library preparation) can produce good and reliable results, especially if the proportion of endogenous DNA in old samples is not extremely low (Cruz-Dávalos et al., 2017). However, when employing very old herbarium specimens (>200 years), a few adaptations to the protocol may help to optimize the efficiency of double-stranded library preparation (Lan and Lindqvist, 2018); for example, it is particularly important to minimize the loss of short endogenous fragments during the purification steps (Fortes and Paijmans, 2015). We tried to achieve this by testing two different modifications to the first purification after the adapter ligation: (1) we used the MinElute PCR purification columns (Qiagen), which are capable of retaining fragments as short as 70 bp; and (2) the standard (magnetic beads–based) purification was performed with an increased volume of beads (1.5× instead of 0.8×). Given that results from the MinElute and from the modified beads-based purification were comparable, we decided to continue with the latter (more cost-effective) method.

In *Xanthium*, 55–65% of the reads mapped to the target regions of the bait kit. These proportions are comparable to those obtained using the same kit with fresh (silica gel–dried) samples (data not published). The target enrichment has already been successfully applied to relatively old herbarium specimens (Hart et al., 2016; Villaverde et al., 2018; Kates et al., 2021); however, for very old specimens (>200 years), methods based on genome skimming and the assembly of multicopy genome regions (e.g., organellar DNA), coupled with single-stranded DNA library preparation, perform better than the target enrichment of single-copy nuclear regions (Bakker, 2017). Our results confirm the potential of the latter technique, even when applied to herbarium specimens up to 200 years old.

Plastome assembly

The generated sequencing reads were used to assemble the plastomes of the archival samples. For the six *Salix* samples, between 5.6% and 11.7% of the reads mapped to the respective references, and it was possible to recover complete plastomes for all samples. This mapping percentage is within the range reported in a recent study of *Salix* plastomes based on non-archival, silica-dried fresh material, for which the percentage of mapped reads varied between 3.1% and 23.5% (Wagner et al., 2021). For *Xanthium*, 0.1–3.1% of reads mapped to the reference, and only 21–83% of the plastome could be recovered. However, target-enrichment library preparation differs from the simple skimming approach, and the assembly of the plastomes was performed based on off-target reads. Under these circumstances, assembling complete plastomes might be difficult. Instead, focusing on the most abundant plastid and nuclear ribosomal regions could be a valuable alternative (Reichelt et al., 2021; Šlenker et al., 2021). Nevertheless, our data support the potential to assemble entire plastid genomes from herbarium samples up to 200 years old using standard extraction and sequencing methods (Bakker, 2017; Alsos et al., 2020).

Concluding remarks

Herbaria harbor huge collections of archival DNA from species that are still underrepresented in phylogenomic studies. Extraction protocols specific for aDNA help to obtain high DNA yields and quality, especially when extracting hDNA from old herbarium specimens; however,

those methods are usually more expensive and time consuming, and require compliance with specific contamination-avoidance procedures not always feasible in standard systematic botany laboratories. The PTB–DTT extraction method presented here takes longer and is more than twice as expensive than a Qiagen DNeasy Plant Mini Kit extraction. Our study showed that it is possible to include herbarium samples from the past two centuries in NGS approaches using standard commercial DNA extraction, library preparation, and target enrichment kits. However, in the case of old (e.g., predating 1900), challenging (e.g., high quantities of secondary compounds, as in the genus *Salix*), or valuable and rare material (e.g., type material and/or scarce herbarium sheets), it might be preferable to use specific aDNA extraction protocols.

AUTHOR CONTRIBUTIONS

S.T. and N.D.W. planned and designed the research. P.M. and S.T. carried out the experimental work. P.M. analyzed the data with the support of the other authors. All authors wrote the first draft and approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Agarose gel images of DNA extracts from the *Salix* and *Xanthium* samples resulting from the PTB–DTT method and the Qiagen DNeasy Plant Mini Kit.

Appendix S2. A comparison of the DNA concentrations (in ng/μL) obtained using the two tested extraction methods for both plant genera. (A, B) DNA concentrations obtained for the (A) *Xanthium* and (B) *Salix* samples extracted using the PTB–DTT and Qiagen DNeasy Plant Mini Kit protocols. Asterisks represent statistically significant differences between the means: (**) $P < 0.01$, (***) $P < 0.001$.

Appendix S3. A comparison of the DNA concentrations (in ng/μL) obtained using the two tested extraction methods for both plant genera in comparison with the age of the samples. (A, B) DNA concentrations obtained for the (A) *Xanthium* and (B) *Salix* samples extracted using the PTB–DTT and Qiagen DNeasy Plant Mini Kit protocols compared against the year of the preparation

of the herbarium sheet. The lines represent a general linear model for the relationship between the DNA concentration and the year of the herbarium sheet for the PTB–DTT and Qiagen Kit protocols. Value *r* represents the calculated Pearson correlation coefficient. Asterisks represent statistically significant linear relationships: (*) $P < 0.05$.

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