EUROPEAN JOURNAL OF ENTOMOLOGY
ISSN (online): 1802-8829
http://www.eje.cz
Eur. J. Entomol. **123**: 48–60, 2026
doi: 10.14411/eje.2026.008
ORIGINAL ARTICLE

Optimization of DNA extraction for insect museomics substantially increases DNA yield

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Key words. Monarch PCR & DNA Cleanup Kit, Monarch Spin PCR & DNA Cleanup Kit, insect collections, whole genome sequencing, endogenous DNA

Abstract. Historical samples from museum and private collections can serve as a time machine, allowing us to follow changes in genetic composition through time as well as obtaining genomic data on past biodiversity. Thus, genetic data from collections (museomics) are increasingly being utilized in scientific studies. However, although several different DNA extraction techniques have been used successfully in insect museomics, direct comparisons between methods are uncommon. It is therefore unclear to what extent simple adjustments of DNA extraction protocols can increase yields. This is especially important when analysing museum specimens that are decades or even hundreds of years old with low endogenous DNA content. Here, we first compared two recommended protocols which include the widely used QIAamp DNA Micro Kit or the Monarch PCR & DNA Cleanup Kit, respectively. We found that the Monarch kit performed substantially better than the QIAamp kit in terms of yield. We then compared various lysis temperatures, the effect of non-destructive versus destructive lysis, and the relative yield from a second round of extraction using the Monarch kit. We evaluated our results both by measurements of DNA concentration and fragment length and results from low coverage whole genome sequencing. We found that a lysis temperature of 42°C performed better than either 56°C or 37°C, using a lysis time of approximately 20 h. Destructive extraction increased yield in some species, and a second round of non-destructive extraction can substantially increase total yield. Finally, we used our selected Monarch kit protocol to extract DNA from legs of 492 additional butterfly specimens (23–128 years old) and 21 small wasp specimens. We observed virtually no effects of specimen age on the amount of DNA extracted or the endogenous DNA content, while older specimens yielded slightly shorter lengths of sequenced fragments. The DNA extraction procedure worked well for specimens up to 128 years of age and we would expect this to be the case for substantially older specimens, which would enable successful DNA extraction from the vast majority of dried insects in collections.

INTRODUCTION

The treasure trove of material in natural history collections is increasingly becoming accessible as a source of material for genomics, although challenges and concerns remain to be addressed (e.g. Short et al., 2018; Weirauch et al., 2020; Raxworthy & Smith, 2021; Jensen et al., 2022). One concern, which is especially relevant for insects due to their small size, is whether enough DNA is retrievable from either a small part of the specimen or through non-destructive* methods. However, the perspectives in getting access to the genetic information within museum specimens are enormous. As many specimens were collected prior to the rapid changes in land use in the 20th and 21st centuries, these specimens can be used to assess the effects of anthropogenic changes on population structure,

* Keeping cuticular structures intact, allowing the specimen or part of specimen extracted from to be returned to the collection. In contrast, for destructive extraction tissues are broken or crushed using various methods.

genetic load and genetic diversity (Diez-del-Molino et al., 2018; Weirauch et al., 2020; Jensen et al., 2022; Brasil et al., 2023; Shpak et al., 2023). Furthermore, as collections often contain specimens from populations which have later gone extinct, DNA from these can shed light on past geographic diversity which may differ from current diversity (e.g. Boe et al., 2022). For phylogenetic and phylogeographic studies, museums contain centuries of collection effort, including specimens of species that are now extinct, globally or locally protected, or otherwise very difficult to collect (Short et al., 2018). Furthermore, it may still be more resource-efficient to use already collected material from natural history collections than to plan and carry out lengthy expeditions to far-away places – even when collection data and other necessary information are sufficient to ensure successful collection activities. Finally, using old type material from collections in genomic studies is highly desirable to re-connect type- and morphology-based taxonomy with DNA-based biodiversity data (e.g. Timmermans et al., 2016).

Obtaining sufficient amounts of DNA from pinned insects, which is the greater part of material in entomological collections, is viewed as particularly challenging (Weirauch et al., 2020). Nevertheless, several different methods and kits have been used successfully for DNA extraction for museomics studies focusing on insects. The various methods and kits used are e.g. DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) (e.g. Lalonde & Marcus, 2020; Mayer et al., 2021, Hsiao et al., 2023), QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) (e.g. Sproul & Maddison, 2017; Gauthier et al., 2020; Twort et al., 2021; Shpak et al., 2023), Qiagen Investigator Kit (Qiagen, Hilden, Germany) (Ferrari et al., 2023), Silica beads (Tin et al., 2014), salting out (McGaughran, 2020), phenol-phenol-chloroform (Gilbert et al., 2007; Thomsen et al., 2009), phenol-chloroform-isoamyl (Parejo et al., 2020), Monarch PCR & DNA Cleanup Kit (New England Biolabs, Ipswich, Massachusetts) (Patzold et al., 2020) and OmniPrep Kit (G-Biosciences, St. Louis, Missouri) (Toussaint et al., 2021; Gauthier et al., 2023). Published comparisons for efficacy of different extraction methods for old insect material are scarce, but see Patzold et al. (2020).

Based on a literature survey and discussions with colleagues, we decided to compare the QIAamp DNA Micro Kit (hereafter QIAamp Kit) and the Monarch PCR & DNA Cleanup Kit (hereafter Monarch Kit) for extracting DNA from single butterfly legs 33–106 years old. The QIAamp Kit has been used successfully in several studies and was also recommended by N. Wahlberg (pers. com.) as their preferred kit as compared to phenol-chloroform, salting out, DNeasy Blood & Tissue Kit, and NucleoSpin Tissue (MACHEREY-NAGEL, Düren, Germany) (Call, 2020). Patzold et al.'s (2020) comparison of DNeasy Blood & Tissue Kit, innuPREP DNA Mini Kit (Analytik Jena, Jena, Germany), and Monarch Kit showed that the Monarch Kit significantly outperformed the two other kits, especially for specimens more than 20 years old, getting on average 17 times more DNA using the Monarch Kit compared to the DNeasy Kit.

In addition to using different DNA extraction methods/kits, various studies have used different modifications of the manufacturers' protocols such as extended lysis time, lowered lysis temperature and extended incubation during elution as well as destructive vs non-destructive extraction (e.g. Krosch & Cranston, 2012; Patzold et al., 2020; Simonsen et al, 2020; Twort et al., 2021). Again, actual comparisons of the effects of these modifications are generally absent from the published literature. Rivero et al. (2004) compared effect of lysis time and temperature for mosquitoes and found that 4 h at 55°C was optimal, but this was done with perfectly fresh and completely homogenized material, and thus their results might not be applicable to museum specimens. Additionally, Cavill et al. (2022) extracted DNA from single legs of museum specimens of bees and showed that re-extracting DNA from the same leg (thus extracting twice from the same piece of tissue) might yield similar amounts of DNA as the first extraction.

To determine the most suitable method for extracting DNA from old (>25 years), dry insect material, we therefore decided to not only compare the QIAamp Kit versus Monarch Kit, but also to assess the effects of lysis temperature, destructive versus non-destructive lysis, and the amount of DNA recovered from a second extraction of the same piece of tissue, the latter as done by Cavill et al. (2022).

MATERIAL AND METHODS

To assess extraction methods and protocols we extracted DNA from legs of 28 specimens of butterflies, between 33 to 106 years old, all from the collections of Natural History Museum Aarhus (NHMA), and all collected in Denmark and registered in NHMA's database (Table S1). The butterfly collections at NHMA are kept at room temperature and ambient humidity and are presently treated with permethrin every third month to prevent damage by insect pests; any treatments prior to 2015 are unknown (H.V. Kristensen, pers. comm.). Furthermore, many of the specimens are donations from private collections, and are thus likely to represent a variety of different initial handling and storage conditions. All direct comparisons were done using two different legs from the same specimens (generally right and left hind leg), except for the comparison of first versus second extraction, in which case the same leg was used for both extractions.

We tested two kits, the QIAamp Kit following the protocol of Twort et al. (2021) and the Monarch Kit following a modified version of the protocol of Patzold et al. (2020) (see Supplement S4 for both protocols). We tested the effect of various modifications (detailed below), but the following was the same for all extractions: Single legs were removed from specimens using forceps cleaned with DNA AWAY and placed in low-bind Eppendorf tubes. The extractions were carried out in a clean-lab facility (Department of Biology, Aarhus University) dedicated to old and environmental DNA. All incubations during lysis were performed overnight (ca. 20 h) in an AccuTherm microtube shaking incubator (Corning Life Sciences, Corning, NY) and centrifugation carried out in an Eppendorf Centrifuge 5424R (Eppendorf, Hamburg, Germany).

Concentration of DNA in each extraction was measured on a Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA) using the

Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA). Fragment length was measured using a TapeStation 4200 (Agilent, Santa Clara, CA) with the D1000 ScreenTape Assay (Agilent, Santa Clara, CA). Total amount of DNA was calculated by multiplying the concentration with the amount of eluate. We obtained 95% confidence intervals by bootstrapping using 'boot' and 'ci.boot' from the boot R package (Davison & Hinkley, 1997; Canty & Ripley, 2024).

DNA extraction protocol optimization

We performed the following comparisons:

(1) Comparison of the amount of DNA extracted using QIAamp Kit following the protocol from Twort et al. (2021) versus Monarch Kit following protocol modified from Patzold et al. (2020). These extractions were non-destructive; the single legs used were not broken or crushed. See Table 1 for a summary of the protocols.

(2) Comparison of the amount of DNA and length of DNA fragments using different lysis temperatures, 56°C vs 42°C, using the Monarch Kit as above, except that agitation during lysis was set to 400 rpm to avoid excessive condensation collecting at the top of the Eppendorf tubes. These extractions were non-destructive.

(3) Comparison of the amount of DNA and length of DNA fragments using of lysis temperatures of 42°C vs 37°C, using the Monarch Kit as in 2. These extractions were non-destructive.

(4) Comparison of the amount of DNA and length of DNA fragments using non-destructive vs destructive extraction, using the Monarch Kit as in 2 and a lysis temperature of 42°C. For the destructive extraction, the legs were broken into pieces inside the Eppendorf tube using a pair of DNA-free forceps.

(5) Comparison of the amount of DNA and length of DNA fragments in 1st versus 2nd extraction of the same leg, using the Monarch Kit as in 2 and a lysis temperature of 42°. These extractions were non-destructive. The legs used in the 1st extraction were immediately placed in new lysis solution (for 2nd extraction) after removing the lysis solution from the 1st extraction.

(6) Comparison of the total amount of DNA from 1st + 2nd extraction (non-destructive) vs destructive extraction, using the Monarch Kit as in 2 and a lysis temperature of 42°C.

(7) Comparison of the amount of DNA and length of DNA fragments using Monarch Kit vs QIAamp Kit for specimens 33–41 years of age using destructive extraction and a lysis temperature of 42°C. For both kits, an incubation time of 10 min was used with the elution buffer. This comparison was carried out as it seemed that the Monarch Kit might be less efficient for specimens <40 years old, see results of comparisons 2 and 3. The test employed the same QIAamp Kit as comparison 1, but incorporated the improvement in lysis temperature (42°C instead of 56°C) for both kits.

Table 1. Summary of the two initial DNA extraction protocols used for comparison. The protocols were based on Twort et al. (2021, QIAamp) and Patzold et al. (2020, Monarch).

	QIAamp Kit	Monarch Kit
Lysis buffer	180 µl Buffer ATL	135 µl Buffer ATL[1]
Proteinase	20 µl Proteinase K	15 µl Proteinase K[1]
Lysis temperature	56°C	56°C
Lysis time	20 h	20 h
Agitation	300 rpm	300 rpm
Binding buffer	200 µl Buffer AL	300 µl Binding Buffer
Ethanol	200 µl absolute ethanol	900 µl absolute ethanol
Wash 1	500 µl Buffer AW1	500 µl DNA Wash Buffer
Wash 2	500 µl Buffer AW2	500 µl DNA Wash Buffer
1st elution	25 µl Buffer AE	20 µl Elution Buffer
Incubation	20 min	10 min
2nd elution	25 µl Buffer AE	20 µl Elution Buffer
Incubation	20 min	10 min

[1] These reagents were from the QIAamp Kit as lysis buffer and Proteinase are not included in the Monarch Kit.

Selected protocol: Extractions and low coverage whole genome sequencing

We also used the selected extraction protocol (Supplement S4 – Extraction protocol, Monarch PCR & DNA Cleanup Kit), both destructive and non-destructive versions (see above) to extract DNA from approximately 500 additional specimens, giving data from a greater number of individuals and species regarding the performance of the selected protocol. These specimens came from NHMA, Natural History Museum Denmark (NHMD), Finnish Museum of Natural History (LUOMUS), Åbo Akademi Collections, Finland (Åbo), and a few specimens from other collections, mainly private. The butterfly collections at NHMD are kept at room temperature and ambient humidity, and no pesticides or fumigants have been applied in the collections since 1966, but prior to this, thymol crystals were placed inside the specimen drawers (T. Pape & N. Scharff, pers. comm.). The butterfly collections at LUOMUS have been kept at 16°C and 40% relative humidity since 2010 with no pesticides or fumigants used (L. Kaila, pers. comm.). Prior to 2010 they were kept at room temperature and ambient humidity, and prior to 1970, mothballs may have been used (L. Kaila, pers. comm.). The butterfly collections at Åbo are kept at room temperature and ambient humidity, and no pesticides or fumigants have been applied in the collections since 1999, but Raid (SC Johnson, Racine, Wisconsin) is sometimes used on donated collections before including these in the main collection (A. Teräs, pers. comm.). Prior to 1999, mothballs (containing naphtalene) were used in the collections (A. Teräs, pers. comm.).

These ≈ 500 additional specimens included several species and families of butterflies: *Aglais urticae* (Linneaus, 1758), 52 specimens; *Hipparchia semele* (Linneaus, 1758), 30 specimens; *Maniola jurtina* (Linneaus, 1758), 61 specimens; *Coenonympha tullia* (Müller, 1764), 51 specimens (Nymphalidae); *Gonepteryx rhamni* (Linneaus, 1758), 63 specimens; *Pieris napi* (Linneaus, 1758), 62 specimens (Pieridae); *Parnassius mnemosyne* (Linneaus, 1758), 30 specimens (Papilionidae); *Thymelicus lineola* (Ochsenheimer, 1808), 51 specimens (Hesperidae); *Lycaena virgaureae* (Linneaus, 1758), 62 specimens; and *Lycaena phlaeas* (Linneaus, 1761), 30 specimens (Lycaenidae). We also used the selected protocol to extract DNA from 21 small wasps (body length 1–2 mm), *Philolema* spp. (Eurytomidae), from the National Museum of Natural History, USA (NMNH), testing the non-destructive version of this protocol on a different group of insects with much smaller body size.

All these specimens, with one or two exceptions, have been successfully sequenced using the SCR library building protocol (Kapp et al., 2021; Grouw et al., 2023) and using 50 bp single end whole genome shotgun sequencing on the DNBSEQ 400 platform at BGI (Shenzhen, China). Some *Aglais urticae* specimens included in the comparisons have also been sequenced using this method, see Table S1. Sequencing data from *A. urticae*, *P. napi*, *H. semele* and *L. phlaeas*, for which reference genomes were publicly available, were mapped to their respective genomes and endogenous DNA content calculated. Genome assemblies were all chromosome level assemblies (all but *P. napi* including mitochondria), soft-masked and downloaded from NCBI [*A. urticae*, assembly GCA_905147175.2 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_905147175.2/ February 2022), *P. napi*, assembly GCF_905475465.1 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_905475465.1/ March 2023), *H. semele*, assembly GCA_933228805.2 (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_933228805.2/ October 2022), and *L. phlaeas*, assembly GCA_905333005.2 (https://www.ncbi.nlm.nih.gov/datasets/taxonomy/282391/ May 2022)] and indexed using BWA index (BWA v0.7.17, Li & Durbin 2009) and SAMtools faidx (SAMtools v1.16.1, Li et al., 2009; Danecek et al., 2021). Raw, single end reads were trimmed using AdapterRemoval v2.3.2 (Schubert et al., 2016), trimming adapters 1: AAGTCGGAGGCCAAGCGGTCTTAGGAAGACAA and 2: AAGTCGGATCGTAGCCATGTCGTTCTGTGAGCCAAGGAGTTG, requiring a minimum Phred quality of 25, a minimum length of 20 bp, and with flags -trimns and -trimqualities enabled. Reads were mapped against their respective genomes using BWA aln with settings previously used for historic samples (-l 16500, -n 0.01, -o 2, Palkopoulou et al., 2015; Kutschera et al., 2022). We then used BWA samse and SAMtools sort to generate alignments in BAM-format. Duplicates were marked and removed using SAMtools markdup, while also filtering for mapping quality (mapQ) ≤ 20, and SAMtools stats was used to generate mapping statistics both prior to and after duplicate and mapQ filtering. Endogenous DNA content was calculated as the percentage of reads remaining after removal of duplicates and MapQ filtering compared to the amount of input reads to the mapping (after the AdapterRemoval step). The mapped sequences (sequencing data from *A. urticae*, *P. napi*, *H. semele* and *L. phlaeas*) are available from European Nucleotide Archive (ENA) as BioProject PRJEB106477.

To check for potential contamination in the reads remaining after removal of duplicates and MapQ filtering, we blasted 100 random reads from one specimen for each species using the specimen with the median number of raw reads for that species. Of these 100 blast searches per specimen, 1–4 reads blasted equally well to another species from the same genus (likely due to sequence similarity), but none of the blast searches found obvious contaminant DNA (e.g. human or microbial DNA).

RESULTS

Monarch Kit vs QIAamp Kit, standard lysis temperature (Comparison 1)

The first comparison, between the QIAamp Kit and the Monarch Kit used 6 specimens 33–71 years old and showed much higher yields (211–1115% increase) for the Monarch Kit for 5 out of 6 samples. However, a single sample (Table 2A, sample 401, *A. cardamines*) yielded only 24% of the DNA retrieved with the QIAamp Kit when using the Monarch Kit. This extraction was the lowest yielding sample of all the test extractions using the Monarch Kit.

Lysis temperature (Comparisons 2 and 3)

The comparisons of lysis temperature each used 5 specimens 36–106 years old. The first comparison of lysis temperature showed a clear improvement in DNA yield when using 42°C compared to 56°C, increasing yield in all cases, with a 115% average increase (Table 2B). The TapeStation readings showed a higher concentration of 60–300 bp fragments at 42°C, visible as a distinct 'shoulder' (extractions 408.2, 409.2, 410.2, 412.2) or a hill (extraction 411.2) for all extractions with a lysis temperature of 42°C, while only extraction 409.1 showed a distinct shoulder with a lysis temperature of 56°C (Fig. 1A).

The second comparison of lysis temperature showed no gain from lowering the lysis temperature further, to 37°C, as this led to a decrease in yield for 4 out of 5 samples compared to 42°C (Table 2C). TapeStation did not show any marked differences between the extracts lysed at 42°C versus 37°C (Fig. 1B).

We did not conduct any direct comparisons between lysis temperatures of 56°C versus 37°C, but the separate assessments used very similar specimens (same species, very similar age of specimens), and the yields using 37°C were generally higher than the yields using 56°C. Comparing the TapeStation results of the extracts also showed a higher concentration of 60–300 bp fragments at 37°C than at 56°C.

Destructive versus non-destructive approach (Comparison 4)

The direct comparison of non-destructive versus destructive extraction used 5 specimens 38–107 years old and showed that breaking the legs nearly doubled the yield (average +90%) and increased the concentration of 60–300 bp fragments, except for specimen 422 which also yielded very similar amounts of DNA from the two extractions (Table 2D, Fig. 2A, B).

First vs second extraction (Comparison 5)

Comparison of DNA yield by re-extracting DNA from the same leg used 5 specimens 38–107 years old and resulted in 24–109% of the yield of DNA compared to the first extraction in 4 out of 5 samples (Table 2E), showing that considerable amounts of DNA remain after the first extraction. On average, the first round of extractions yielded 89 ng DNA, while the second round yielded 34 ng. TapeStation measurements showed lower concentrations of fragments of 60–300 bp for most 2nd elutions, except for specimen 421, although the second extraction yielded less DNA than the first extraction (Fig. 2B, C).

Destructive extraction versus extracting twice (Comparison 6)

Comparison of the amount of DNA recovered using two non-destructive extractions versus destructive extraction (using the same 5 specimens as in Comparisons 4 and 5) showed that destructive extraction yielded more DNA for 4 out of 5 specimens, on average yielding 26% more DNA (Table 2F). However, the difference in total yield using two extractions was much decreased compared to destructive versus a single non-destructive extraction.

Monarch Kit vs QIAamp Kit, optimized lysis temperature (Comparison 7)

The second comparison of the Monarch versus QIAamp Kit, focused on specimens about 30–40 years old, as these had given lower yields in in some of the previous assessments (comparisons 2–5). This comparison showed consistently higher yields (average +242%) for the Monarch Kit compared to the QIAamp Kit, both for the 5 specimens from the 1980's and the two control specimens from around 1950 (Table 2G). TapeStation results showed similar relative concentrations of 60–300 bp fragments between the two kits (Fig. 3).

Table 2. Result from the paired extractions (using single matching legs from the same individual butterflies except in E) comparing the efficiency of various DNA extraction procedures. In all cases, ng/µl denotes the DNA concentration in the eluate (QIAamp Kit: 50 µl eluate. Monarch Kit: 40 µl eluate). Total DNA denotes the total amount of DNA extracted. Percent change denotes the change in total amount of DNA extracted. B–F used the Monarch Kit. A – QIAamp Kit versus Monarch Kit. B – Lysis temperature of 56°C vs 42°C. C – Lysis temperature of 42°C vs 37°C. D – Non-destructive (leg not broken or crushed) versus destructive (leg broken). E – First versus second extractions re-extracting from the same leg. F – Two rounds of non-destructive (leg not broken or crushed) versus destructive (leg broken). G – QIAamp Kit versus Monarch Kit for younger specimens (most 34–42 years) using a lysis temperature of 42°C.

A: Comparison 1

Extr. #	Species	Collection date	QIAamp Kit		Monarch Kit		Percent change
			ng/µl	Total DNA	ng/µl	Total DNA	
397	*Satyrium w-album*	10-07-1940	0.390	19.5	1.57	62.8	+222%
398	*Aglais urticae*	03-08-1951	0.100	5.17	1.57	62.8	+1115%
399	*Aglais io*	06-08-1949	0.157	7.86	2.30	92.1	+1072%
400	*Satyrium w-album*	20-07-1980	0.517	25.9	2.01	80.6	+211%
401	*Anthocharis cardamines*	17-05-1986	0.496	24.8	0.146	5.83	–76%
402	*Boloria euphrosyne*	27-05-1988	0.331	16.6	4.60	183.8	+1007%
Total DNA, mean			16.6		81.3		
Total DNA, 95% confidence interval			10.4–23.0		34.5–123.5		

B: Comparison 2

Extr. #	Species	Collection date	56°C		42°C		Percent change
			ng/µl	Total DNA	ng/µl	Total DNA	
408	*Aglais urticae*	1915	2.51	100.4	3.84	153.8	+53%
409	*Aglais urticae*	1915	3.40	136.1	3.92	156.8	+15%
410	*Aglais urticae*	Sept 1947	0.516	20.6	1.31	52.3	+154%
411	*Aglais urticae*	Sept 1947	1.03	41.2	4.24	169.6	+312%
412	*Aglais urticae*	12-08-1985	0.572	22.9	0.815	32.6	+42%
Total DNA, mean			64.2		113.0		
Total DNA, 95% confidence interval			18.5–102.8		64.7–161.3		

C: Comparison 3

Extr. #	Species	Collection date	42°C		37°C		Percent change
			ng/µl	Total DNA	ng/µl	Total DNA	
413	*Aglais urticae*	1915	4.23	169.0	5.59	223.6	+32%
414	*Aglais urticae*	1915	4.05	162.2	3.19	127.5	–21%
415	*Aglais urticae*	26-07-1949	2.21	88.5	1.25	49.8	–44%
416	*Aglais urticae*	24-07-1951	5.49	219.6	4.07	162.9	–26%
417	*Pieris napi*	31-05-1985	2.50	100.0	1.98	80.1	–20%
Total DNA, mean			147.9		128.8		
Total DNA, 95% confidence interval			107.8–186.5		72.5–179.1		

D: Comparison 4

Extr. #	Species	Collection date	Non-destructive		Destructive		Percent change
			ng/µl	Total DNA	ng/µl	Total DNA	
418	*Aglais urticae*	1915	3.03	121.2	5.45	218.0	+80%
419	*Aglais urticae*	1915	3.37	134.9	5.37	214.8	+59%
420	*Aglais urticae*	01-08-1949	0.818	32.7	2.33	93.2	+185%
421	*Aglais urticae*	02-08-1951	2.06	82.2	4.52	180.8	+120%
422	*Pieris napi*	08-07-1984	1.85	73.9	1.92	76.8	+4%
Total DNA, mean			89.0		156.7		
Total DNA, 95% confidence interval			56.3–124.7		104.2–209.3		

E: Comparison 5

Extr. #	Species	Collection date	1st extraction		2nd extraction		Percent change
			ng/µl	Total DNA	ng/µl	Total DNA	
418	*Aglais urticae*	1915	3.03	121.2	1.20	48.0	–60%
419	*Aglais urticae*	1915	3.37	134.9	0.805	32.2	–76%
420	*Aglais urticae*	01-08-1949	0.818	32.7	0.895	35.8	+9%
421	*Aglais urticae*	02-08-1951	2.06	82.2	1.22	48.7	–41%
422	*Pieris napi*	08-07-1984	1.85	73.9	0.153	6.13	–92%
Total DNA, mean			89.0		34.2		
Total DNA, 95% confidence interval			56.3–124.7		22.5–47.8		

F: Comparison 6

Extr. #	Species	Collection date	Non-destructive	Destructive	Percent change
			Total DNA 1st + 2nd extr.	Total DNA	
418	*Aglais urticae*	1915	169.2	218.0	+29%
419	*Aglais urticae*	1915	167.1	214.8	+29%
420	*Aglais urticae*	01-08-1949	68.5	93.2	+36%
421	*Aglais urticae*	02-08-1951	130.9	180.8	+38%
422	*Pieris napi*	08-07-1984	80.03	76.8	–4%
Total DNA, mean			123.1	156.7	
Total DNA, 95% confidence interval			85.6–160.7	104.2–209.3	

G: Comparison 7

Extr. #	Species	Collection date	QIAamp Kit		Monarch Kit		Percent change
			ng/µl	Total DNA	ng/µl	Total DNA	
423	*Aglais urticae*	28-08-1984	1.40	78.5	6.25	280.0	+257%
424	*Pieris napi*	08-07-1984	0.295	16.5	0.94	42.0	+155%
425	*Satyrium w-album*	24-07-1980	0.518	29.0	1.83	82.0	+183%
426	*Pieris rapae*	27-08-1987	0.499	28.0	2.31	103.6	+270%
427	*Aglais urticae*	14-08-1988	0.991	55.5	5.05	226.0	+308%
428	*Aglais urticae*[1]	08-08-1949	0.982	55.0	4.55	203.6	+271%
429	*Aglais urticae*[1]	30-07-1951	0.881	49.4	3.86	172.8	+251%
Total DNA, mean			44.6		158.6		
Total DNA, 95% confidence interval			30.5–59.0		101.9–217.3		

[1] Control

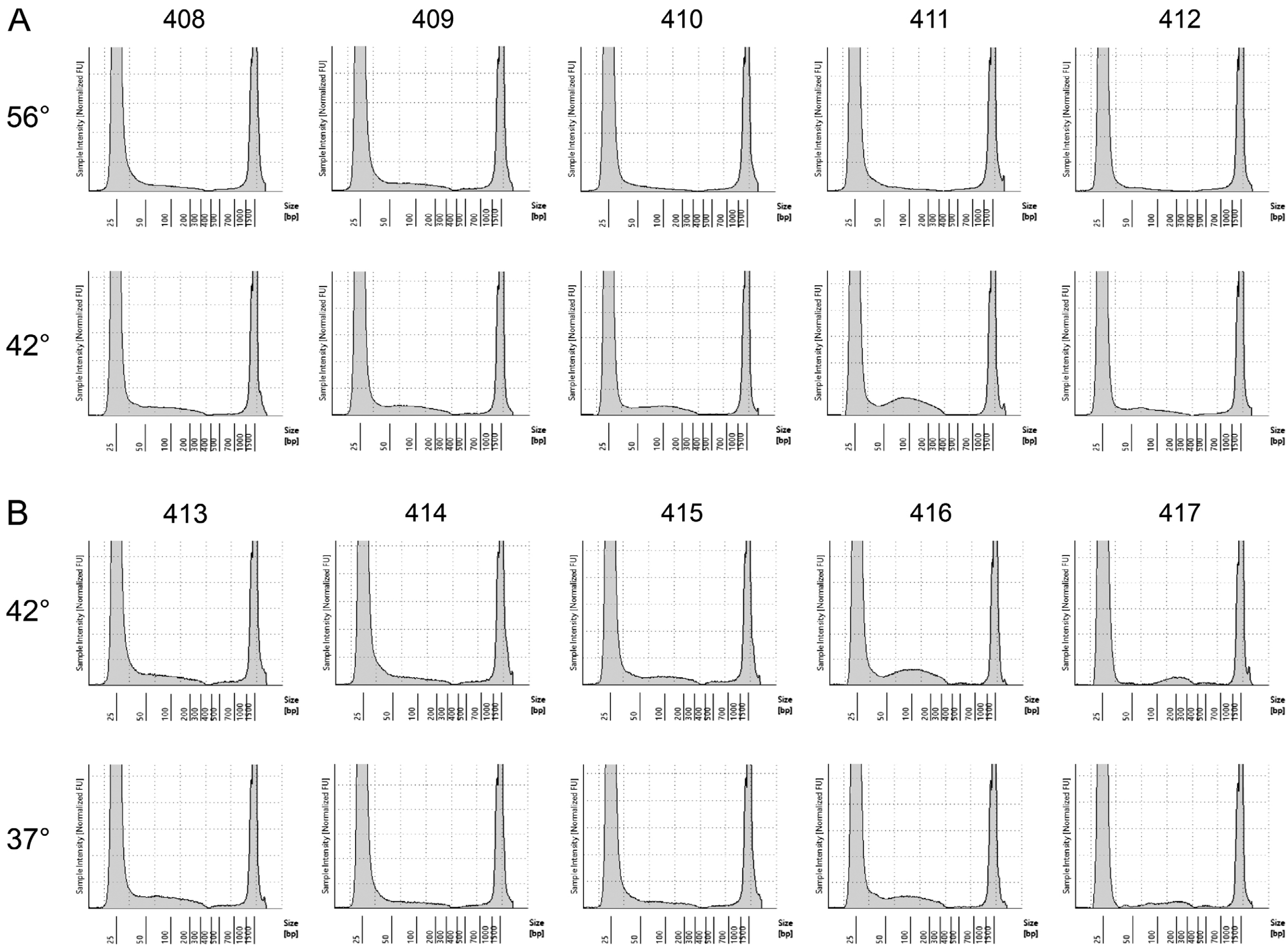

Fig. 1. TapeStation graphs of DNA extraction eluates using lysis temperatures of 56°C versus 42°C (A) and 42°C versus 37°C (B). Each set of comparisons used legs from the same specimens: A – 56°C, left hind leg; 42°C, right hind leg. B – 42°C, left hind leg; 37°C, right hind leg. The numbers above each set of graphs denote the specimen.

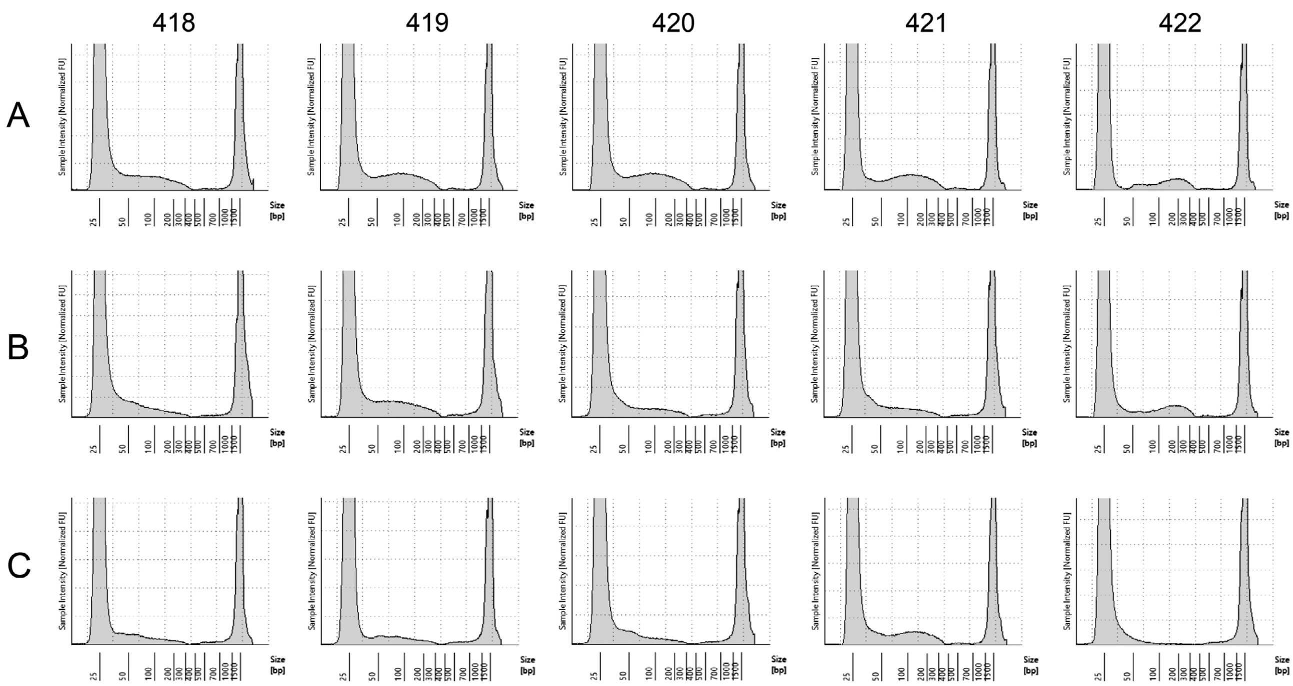

Fig. 2. TapeStation graphs of DNA extraction eluates from destructive extraction (leg broken) versus non-destructive (leg not broken) 1st and 2nd extraction. The three sets of extractions were performed on legs from the same specimens (Destructive: left hind leg. Non-destructive: right hind leg). A – Destructive extraction. B – Non-destructive extraction. C – Second round of non-destructive extraction using the same legs as used in B. The numbers above each set of graphs denote the specimen. Please note that 418–421 were all *A. urticae*.

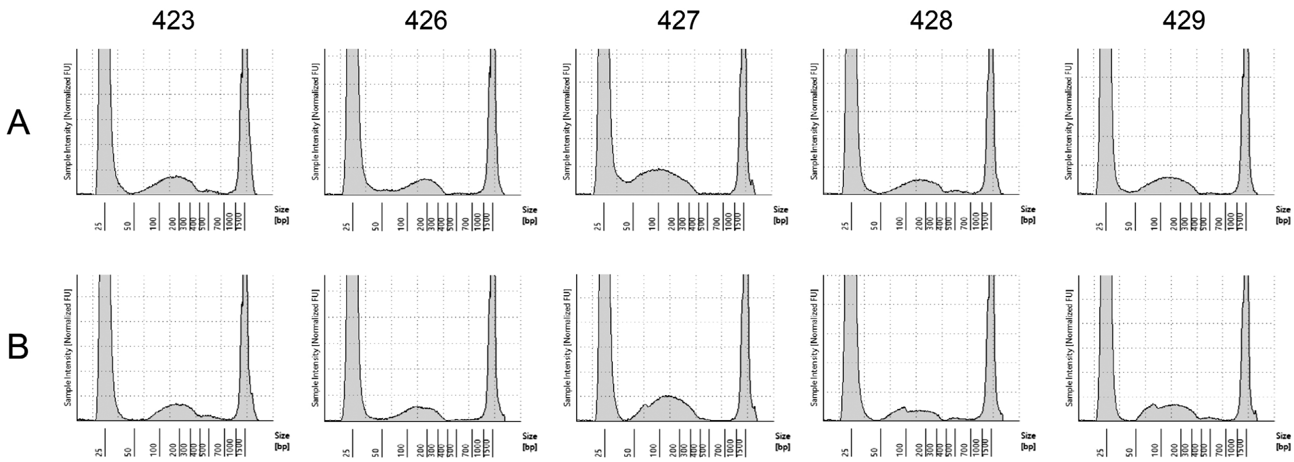

Fig. 3. TapeStation graphs of DNA extraction eluates from extraction using Monarch kit versus QIAamp kit using the selected lysis conditions (42°C for 20 h). The two sets of extractions were performed on legs from the same specimens (Monarch: left hind leg. QIAamp: right hind leg). The numbers above each set of graphs denote the specimen. TapeStation graphs for 424 and 425 are not included in the figure, as TapeStation graphs are not available for both legs due to misloading of the TapeStation machine.

DNA yield and effect of specimen age

Following selection of the final protocol, we extracted DNA from hundreds of legs of pinned museum specimens of butterflies, showing more than a four-fold difference in the amounts of DNA in legs of different species (Table 3). These extractions also revealed that the effect of non-destructive vs. destructive extraction is highly species-dependent, as destructive extraction greatly increased yields in *A. urticae* and *G. rhamni* but seemed to have very little or a small negative effect in other species (*M. jurtina*, *C. tullia*, *T. lineola*, *L. virgaureae*).

We also used the selected protocol to extract DNA from *Philolema* spp. wasps (whole body), getting good DNA yields, showing that this method also works well for other insects than butterflies. The wasp specimens were 6–77 years old and yielded on average 109 ng of DNA per specimen (spread 9–310 ng).

Using the selected extraction protocol, we found virtually no overall effect of specimen age on DNA yield (ng DNA obtained in an extraction, Fig. 4A), but with some variation between species (Table 3). Likewise, we found no effect of age on endogenous DNA content (percentage of sequenced reads left after filtering and mapping) in specimens that were sequenced and mapped to their corresponding reference genomes (Tables 4, S2, Fig. 4B). However, we found a noticeable effect of age on read length of sequenced fragments, as average read length decreased from approximately 44 bp for specimens 23–25 years old to 37 bp for specimens around 110 years old (Fig. 4C).

We found a strong effect of specimen size on DNA yield, especially with regard to the destructive DNA extractions (Table 5). Within the non-destructive extractions, *G. rhamni* was an outlier, yielding less DNA than expected from its size. As *G. rhamni* was also the species with the largest relative difference in yields from destructive versus non-destructive extractions (Table 3), the relative inefficiency of non-destructive extraction for this species likely explains the lower-than-expected yields of these extractions.

Table 3. DNA extracted from single legs of Lepidoptera from Denmark (DK) and Finland (FIN) using the selected protocol. The data on specimen size are averages of male and female size from Middleton-Welling et al. (2020). The slope and R^2 values are based on a best fit line calculated using linear regression. A negative slope value indicates a positive correlation between specimen age and DNA yield.

Species, extraction type, country of origin	N	Specimen age (years)	Specimen size (mm)		DNA amount (ng)		Effect of age	
			Forewing length	Wingspan	Range	Average	Slope	R^2
Aglais urticae, destructive, DK	12	67–112	23.5	46.5	123–368	225	–0.8809	0.0599
Aglais urticae, non-destructive, DK	40	33–112	23.5	46.5	8.5–236	104	–0.4229	0.0459
Hipparchia semele, non-destructive, FIN	30	25–118	27.5	48.25	24–386	137	–0.9471	0.1008
Maniola jurtina, destructive, DK	13	68–111	24.5	40.75	25–252	115	–2.3363	0.3299
Maniola jurtina, non-destructive, DK	48	34–115	24.5	40.75	25–375	134	0.7335	0.0701
Coenonympha tullia, destructive,DK	13	31–88	19.75	29.75	51–200	94	0.1954	0.0081
Coenonympha tullia, non-destructive,DK	38	34–114	19.75	29.75	32–215	93	–0.5558	0.1283
Pieris napi, non-destructive, DK	62	34–113	22.5	41	1.7–140	56	0.2052	0.0375
Gonepteryx rhamni, destructive, DK	18	40–109	28.23	58	43–296	143	–0.3230	0.0103
Gonepteryx rhamni, non-destructive, DK	45	31–117	28.23	58	8.2–122	38	0.1222	0.0182
Parnassius mnemosyne, destructive, DK	21	65–118	32.5	57	67–317	205	0.6695	0.0459
Parnassius mnemosyne, non-destructive, DK	9	64–111	32.5	57	3.8–274	168	–1.5532	0.1821
Thymelicus lineola, destructive, DK	16	35–108	12.24	24.75	26–135	67	–0.5910	0.1409
Thymelicus lineola, non-destructive, DK	35	32–112	12.24	24.75	9.7–144	63	0.1772	0.0251
Lycaena virgaureae, destructive, DK	12	34–117	16.39	27.25	28–67	42	–0.0249	0.0073
Lycaena virgaureae, non-destructive, DK	50	31–115	16.39	27.25	17–199	67	0.2831	0.0550
Lycaena phlaeas, non-destructive, FIN	30	23–128	13.5	25.5	25–132	63	–0.0465	0.0031

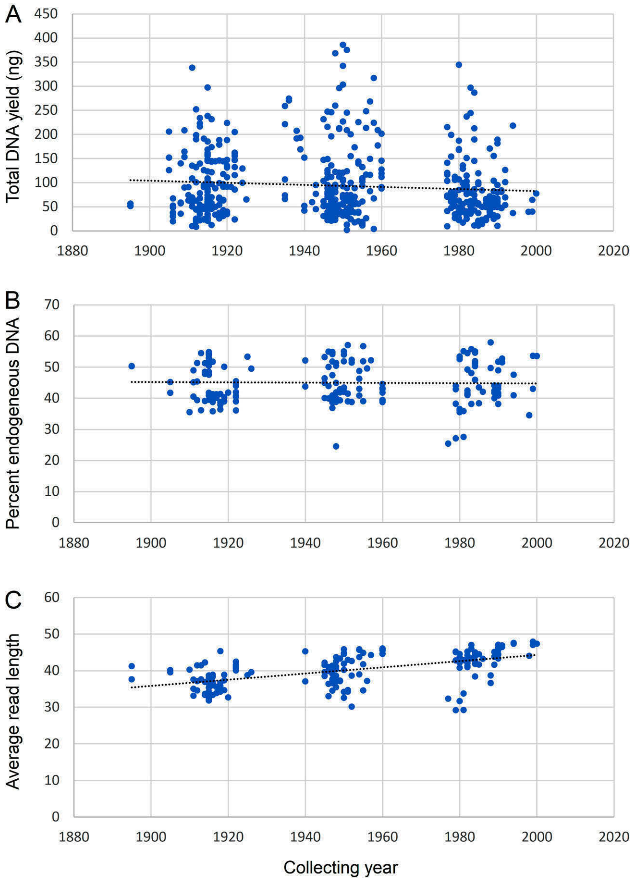

Fig. 4. Effect of age on extracted DNA. A – Age has little effect on DNA yield. Although yield increases slightly with time since death, R^2 is only 0.007. Amount of extracted DNA from *A. urticae*, *H. semele*, *M. jurtina*, *C. tullia*, *P. napi*, *G. rhamni*, *P. mnemosyne*, *T. lineola*, *L. virgaureae* and *L. phlaeas* plotted against collection year, with a best fit line calculated using linear regression ($y = –0.2133x + 509.34$ and $R^2 = 0.0074$, n = 498). B – The percentage of endogenous DNA does not vary with time since death. Percentage of endogenous DNA from *A. urticae*, *H. semele*, *P. napi* and *L. phlaeas* plotted against collection year, with a best fit line calculated using linear regression ($y = –0.0041x + 53.011$ and $R^2 = 0.0003$, n = 174). C – The average read length decreases with time since death. Average sequenced read length for from *A. urticae*, *H. semele*, *P. napi* and *L. phlaeas* plotted against collection year, with a best fit line calculated using linear regression ($y = 0.0846x – 124.93$ and $R^2 = 0.2989$, n = 174). Note that maximum sequenced read length was 50 bp and minimum mapped read length was 20 bp.

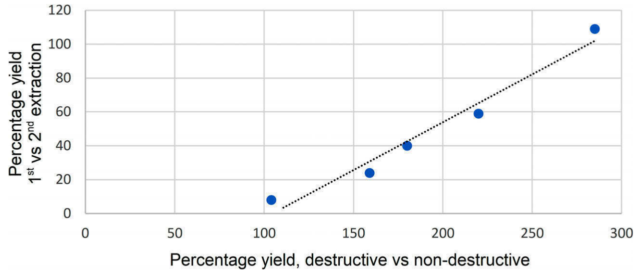

Fig. 5. Relative yields of 2nd lysis vs destructive extraction show strong correlation ($R^2 = 0.9654$). The comparison was done using pairs of legs from five specimens. The yields shown here are relative to amount of DNA from a 1st non-destructive lysis with a best fit line calculated using linear regression ($y = 0.5652x – 59.161$, n = 5).

DISCUSSION

Monarch Kit vs QIAamp Kit

Our results confirm the findings of Patzold et al. (2020) that the Monarch Kit performs better than standard methods, such as the DNeasy Blood & Tissue Kit (Patzold et al., 2020) and the QIAamp Kit (this study) when extracting DNA from museum material. In the two direct comparisons, we found that the Monarch Kit yielded on average 592% (Table 2A, Comparison 1, N = 6) and 242% (Table 2G, Comparison 7, N = 7) more DNA than the QIAamp Kit while Patzold et al. (2020) found that the Monarch Kit yielded 1625% more than the DNeasy Kit in a direct comparison (N = 6). Although Patzold et al. (2020) and this study compared the Monarch Kit to two different extraction kits, these two kits are quite similar: The QIAamp Kit and the DNeasy Kit contain the same reagents, and the respective protocols for isolation of DNA from animal tissues use the same amounts except for the elution buffer (Qiagen, 2014, 2023), thus any difference between the kits is likely due to the spin columns. Another difference between this study and Patzold et al. (2020) is that Patzold et al. (2020) used lysis buffer FN (AGOWA, now LGC Biosearch Technologies, Hoddesdon, UK) and proteinase K from Analytik Jena (Jena, Germany) while we used Buffer ATL and proteinase K from Qiagen with the Monarch Kit.

Part of the differences in DNA yield between the Monarch and QIAamp/DNeasy Kits is likely caused by differences in spin column design. The diameter of the silica membrane is much smaller in the Monarch columns than in the QIAamp/DNeasy columns (see New England Biolabs, 2025 for a visual comparison). Thus, the same volume of elution buffer applied to the membrane in a Monarch or QIAamp/DNeasy column gives a higher elution buffer volume per mm² of filter in a Monarch column. Total DNA

Table 4. Raw sequencing reads, mapped reads, MapQ > 20 reads and percentage of post-filtering endogeneous DNA content in specimens sequenced using SCR library build and single end 50 bp sequencing. Endogenous DNA content was calculated as the percentage of reads remaining after MapQ ≥ 20 filtering and removal of duplicates in relation to the amount of input reads to the mapping (after the AdapterRemoval step). The slope and R^2 values are based on a best fit line calculated using linear regression. See Table S2 for information about individual specimens.

Species	N	Age (years)	Mill. raw reads		Mill. mapped reads		Mill. MapQ > 20[1]		% endogeneous DNA		Effect of age	
			Range	Average	Range	Average	Range	Average	Range	Average	Slope	R^2
Aglais urticae	57	33–112	14.1–202.3	98.8	6.5–139.1	60.6	3.0–74.7	35.9	24.5–57.9	49.8	–0.0094	0.0018
Hipparchia semele	30	25–118	58.0–139.5	97.3	42.7–103.6	71.3	22.8–55.8	38.5	34.5–47.5	41.8	–0.0085	0.0085
Pieris napi	61	34–113	42.5–168.7	95.8	11.7–123.0	63.4	5.0–61.4	31.7	25.4–43.9	39.4	–0.0034	0.0008
Lycaena phlaeas	30	23–128	44.9–129.1	90.7	34.0–105.5	71.4	21.3–61.1	41.9	37.9–55.8	49.5	0.0172	0.0144

[1] MapQ > 20 is exclusive of duplicates.

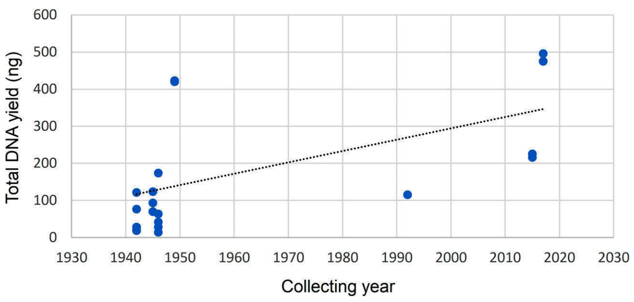

Fig. 6. Age of specimens is correlated with decreased DNA yield comparing samples collected 2015–2017 and older samples. Amount of extracted DNA from small wasps (*Philolema* spp.) plotted against collection year, with a best fit line calculated using linear regression (y = 3.063x – 5831.4 and R^2 = 0.2859, n = 21).

yield will generally be higher when using a larger elution volume (but concentration will be lower) (New England BioLabs, 2021; Qiagen, 2023), hence the larger volume of elution buffer per mm² membrane in the Monarch columns may contribute to the higher yields of DNA. Another difference is that the Monarch columns are designed without frits (a filter underneath the silica membrane that holds it in place), while Qiagen's (the manufacturer of the QIAamp and DNeasy kits) columns do have frits (New England Biolabs, 2025). Frits can cause retention of small droplets of buffer (New England Biolabs, 2025), and if elution buffer is retained this could cause less complete elution when using the QIAamp or DNeasy columns compared to the Monarch columns. The QIAamp DNA Micro Kit Handbook (Qiagen, 2014) states that the volume of eluate will be up to 5 µl less than the volume of elution buffer used. Our own observations using the Monarch Kit is that the eluate volume is equal to the volume of elution buffer used (even a 1 µl difference per elution would be quite noticeable when eluting twice in 20 µl). Hence, the Monarch columns appear to offer a more complete elution, which may also positively affect DNA yield. Another difference is in the protocols. The protocols used with the Monarch Kit by both Patzold et al. (2020) and in the present study are optimized for short fragments of DNA while the DNeasy (used by Patzold et al., 2020) and QIAamp (used in the present study) protocols are optimized for much longer fragments. Thus, they seem less appropriate for old samples that are often highly fragmented (Raxworthy & Smith, 2021). See a detailed discussion of this below (in the section Effect of specimen age).

Lysis temperature

Some studies have reported successful extractions using a lower lysis temperature on older insect material (e.g. Krosch & Cranston, 2012; Simonsen et al., 2020), but did not provide any direct comparison of yields using lower lysis temperatures instead of the standard 55–56°C. We found that lowering the lysis temperature to 42°C increased the yield by 115% on average (Table 2B, Comparison 2, N = 5). However, lowering the lysis temperature further, to 37°C, decreased yield for most samples (average –16%) compared to using 42°C (Table 2C, Comparison 3, N = 5). We did not perform any direct comparison of 56°C versus 37°C, but when looking at yields from comparable specimens (same species, similar ages), 37°C seems to work better than 56°C (Table 2B, C).

This contrasts with the results of Rivero et al. (2004) who found that 55°C yielded 30–100% more DNA than 42°C after 16 h of lysis. However, Rivero et al. (2004) was working with fresh and homogenised material, compared to our material which was between 36 and 106 years old, and not specifically preserved for DNA extraction. Thus, it is possible that the standard lysis temperature is optimal for fresh, homogenised material, while a lower lysis temperature is optimal for the older, dried material present in museum collections. One possibility is that lysis enzymes are optimized for working at around 55°C for a relatively short period of time (2–4 h), but might be subjected to gradual heat denaturation and thus losing efficiency over time. A lower lysis temperature likely gives a reduced lysis efficiency during the first hours, but if the enzyme stays active for longer, and the longer lysis time allows better penetration of dried tissues, this might explain the difference.

In addition to increasing the amount of extracted DNA, a lower lysis temperature also seems to increase the quality of the extracted DNA (concentration of fragments of 60–300 bp), independently of the amount of DNA extracted, see Fig. 1 and Table 2B, C.

Destructive versus non-destructive approach

In the direct comparison, destructive extraction yielded substantially more DNA for four out of five specimens (Table 2D, Comparison 4). However, the several hundred subsequent extractions using the selected protocol strongly suggest that this result was dependent on the species used, as the effect (or lack thereof) of destructive versus non-destructive extraction was highly species-specific: In the direct comparison, the four specimens showing improved DNA yield using destructive extraction were *A. urticae* while the specimen showing no increase was *P. napi*. In the post-optimization extractions, *A. urticae* was one of two species (out of seven) showing a substantial increase in DNA yield from destructive versus non-destructive extractions (Table 3).

Differences in amount of extracted DNA could conceivably be caused by differences in storage conditions between collections (see discussion in Patzold et al., 2020), and the seven species for which legs were subjected to both destructive and non-destructive extraction were generally from two different collections (destructive: NHMD; non-destructive: NHMA). However, if the amount of extracted DNA was dependent on differences in storage conditions between NHMD and NHMA, we should have seen this difference across the seven species, not just in two of

Table 5. Correlation between specimen size and DNA yield. The slope and R^2 values are based on a best fit line calculated using linear regression. For specimen size and DNA yield, see Table 3.

Extraction type	N (no. of species)	Forewing length		Wingspan	
		Slope	R^2	Slope	R^2
Destructive	7	7.5403	0.5847	3.9679	0.6487
Non-destructive	10	3.7793	0.3451	1.3492	0.161

them. Thus, a difference between species in ease of non-destructive DNA extraction seems the most likely explanation. This difference between *A. urticae* and *G. rhamni* and the other included species could be related to their life history. *Aglais urticae* and *G. rhamni* overwinter as adults while the other species overwinter as either eggs, larvae or pupae. This difference in life history might be related to physiological adaptations which make their cuticle more resistant to the enzymatic activity of Proteinase K, hampering DNA extraction when the cuticle hasn't been physically breached.

First vs second extraction

We found that most samples still contained substantial amounts of DNA after being extracted once (non-destructively) with the second round of extractions yielding on average 48% as much DNA as the 1st round (Table 2E, Comparison 5, N = 5). This is consistent with the results from Cavill et al. (2022) who found that the first round of extraction yielded <2.25–18.98 ng DNA while the second round yielded <2.25–14.4 ng DNA (on average ≈75% of the 1st round), although the number of samples yielding measurable amounts of DNA (>0.05 ng/µl) decreased from 6/8 to 3/8. We did not sequence both 1st and 2nd extractions from the same specimen and thus did not directly assess whether the endogenous DNA content (percentage of sequenced reads left after filtering and mapping) between the two extractions is similar. However, Cavill et al. (2022) found that while the endogenous DNA content was lower in the 2nd round of extractions, the endogenous DNA content was relatively similar between the 1st and 2nd extractions within samples.

It is worth noting that we found a perfect qualitative correlation between those specimens giving the proportionally greatest yields from the second extraction and those showing the largest increase between non-destructive and destructive extraction (Fig. 5). This strongly indicates that legs from these specimens for some reason were difficult to lyse adequately, and thus that 20 h of lysis of an unbroken leg left substantial amounts of DNA still in the sample. This DNA can be partially captured by subjecting the sample to a second round of extraction, but for most samples, two rounds of non-destructive extraction only yielded about 75% of the DNA compared with destructive extraction (Table 2F, Comparison 6, N = 5). However, the 2nd extraction provided a substantial increase in the amount of total DNA for most of the samples. Thus a 2nd round of extraction is relevant when it is necessary to maximise DNA yield while keeping the sample intact.

DNA yields compared to previous studies

On average, we retrieved much lower amounts of DNA (total DNA 1.7–386 ng) than Patzold et al. (2020, total DNA 33–1106 ng) using the Monarch Kit. However, Patzold et al. (2020) used single legs from hawkmoths (Sphingidae), some of the largest Lepidoptera, and noted that the amounts of extracted DNA might be lower from other (non-sphingid), smaller, lepidopterans. This is supported by our results as we found a strong correlation between size of a species (measured as either forewing length or wingspan) and the average amount of DNA retrieved from a single leg, especially for the destructive extractions (Table 5).

We retrieved larger, but comparable, amounts than Gauthier et al. (2020) did from single legs of butterflies [*Erebia embla* (Thunberg, 1791) and *Lyceaena helle* (Denis & Schiffermüller, 1775)] of a similar size to our species. On the other hand, we retrieved much larger amounts of DNA than Cavill et al. (2022) did from single legs of Asian honeybees (*Apis cerana* Fabricius, 1793, total DNA <2.25–19 ng), although the legs are of similar size to the Lepidoptera legs we extracted DNA from. See Table S3 for DNA yields from old insects in various studies.

Effect of specimen age

Several rounds of extractions with the Monarch Kit during the testing phase yielded lower amounts of DNA for newer specimens (33–41 years old). Furthermore, Patzold et al. (2020) reported that with the Monarch Kit, the amount of extracted DNA was slightly higher from older samples. Patzold et al. (2020) explained this as the result of Monarch Kit protocol being less efficient at extracting dsDNA compared to ssDNA, the latter being more prevalent in older samples. To assess whether the QIAamp Kit might be better for samples less than 50 years old, we made a direct comparison between the two kits focused on specimens collected in the 1980's, incorporating the improvement we had made to the lysis conditions. We also included two specimens collected around 1950 as controls (Table 2G, Comparison 7, N = 5+2). In this comparison, the Monarch Kit consistently recovered much more DNA than the QIAamp Kit (average increase 242%), with no discernible difference between newer and older specimens. Furthermore, in the hundreds of extractions subsequently made using the selected protocol, we did not find any overall effect of specimen age on DNA yield (Fig. 4A). The low DNA yields from specimens from the 1980's found in the testing phase were in several cases a *P. napi* from the 1980's being compared to older *A. urticae* specimens. Data from the subsequent extractions using the selected protocol show that on average single legs of *A. urticae* yields 2–4 times as much DNA as single legs of *P. napi* (Table 3), providing a plausible explanation for why lower yields were observed in more recent specimens, unrelated to the efficacy of the extraction method.

Other studies have found large negative effects of age on the DNA yield from pinned insects using other kits. Ferrari et al. (2023) retrieved much lower maximum yields for specimens more than 25 years old with a further decline for specimens more than 70 years old using the Qiagen Investigator Kit. Patzold et al. (2020) retrieved much lower maximum yields for specimens more than 20 years old using the DNeasy Blood & Tissue Kit. The data from Gauthier et al. (2023) shows a sharp decline in maximum DNA yields for specimens more than 25 years old, but yields appear stable for specimens 25–123 years old using the OmniPrep Kit.

As our youngest Lepidoptera specimens were 23 years old, we have no data on what maximum DNA yields using the Monarch Kit would be on Lepidoptera specimens younger than this (e.g. fresh or <10 years old). However, four of the wasp specimens were collected in 2015–2017 while the majority were collected during 1942–1949. The older specimens show a decline in both maximum and average amount of DNA extracted compared to the recently collected specimens, but as only a single specimen was collected between these two time periods (in 1992), it is difficult to pinpoint when the decline in extracted DNA happens (Fig. 6). Our results are thus compatible with those of Gauthier et al. (2023), but we found no decline in DNA yield from specimens more than 70 years old, contrasting with the findings of Ferrari et al. (2023).

One reason for the stable DNA yields (ng DNA obtained in an extraction) with respect to specimen age in this study as well as Patzold et al. (2020) is likely that the relative amounts of binding buffer and ethanol used in the extraction protocol is optimized for short fragments and single stranded DNA (≥15 bp dsDNA, ≥18 nt ssDNA) (Patzold et al., 2020; New England Biolabs, 2021). Other silica column-based protocols are optimized for longer fragments, e.g. DNeasy Blood & Tissue Kit and QIAamp Mini Kit (both Qiagen) are optimized for 30 kb fragments, range 100 bp to 50 kb, and at least for the QIAamp Mini Kit, recovery efficiency declines below 200 bp (Qiagen, 2024a, b, c).

We found virtually no effect ($y = -0.0041x$) of age on proportion of endogenous DNA content (percentage of sequenced reads left after filtering and mapping) in our samples (Fig. 4B). The species for which reference genomes were available yielded 25–58% (average 45%) endogenous DNA, comparable with results from other studies using single legs of insects, see Tables 4 and S3. However, Mullin et al. (2022) found a negative effect of specimen age on the proportion of endogenous DNA, and the data from Korlevic et al. (2021) show the same pattern. The age range of our samples is slightly smaller than in Mullin et al. (2022) but larger than in that of Korlevic et al. (2021), so it is not clear what causes the difference in the found effect of age on percentage endogenous DNA. However, it is possible that if the extraction method used is optimized for longer fragments, and the length of the endogenous DNA fragments decrease with age, a larger proportion of contaminant DNA (if this is more recent and thus consists of longer fragments) is getting extracted from older specimens. This would provide a potential explanation for the differences found in the effect of specimen age on the amount of endogenous DNA between this study, Korlevic et al. (2021) and Mullin et al. (2022).

While we found only negligible effects of age on the amount of DNA extracted or endogenous DNA content on our butterfly leg samples (23–128 years old), we did find a notable effect on sequenced read length. Read length decreased from approximately 44 bp for specimens 23–25 years old to 37 bp for specimens around 110 years old. The difference in average fragment length in the extracts is likely greater than this as we only sequenced 50 bp and as reads of less than 20 bp were filtered out, and thus not included in the calculation of average read length. However, none of our specimens have average read lengths close to the lower cutoff (20 bp), and we found virtually no effect of age on endogenous DNA content, thus we expect the procedure to work for specimens substantially older than the oldest specimens included here. If we assume that sequenced read length decreases by 0.085 bp per year, and that average read length is 37 bp at 110 years of age (Fig. 4C), we would expect an average sequenced read length of 25 bp at 250 years of age. At this point a much larger percentage of fragments would be removed with a 20 bp cutoff, but it should still be possible to obtain useful data. As very few pinned insects are likely to be more than 250 years old (Raxworthty & Smith, 2021, pers. obs. by MD and TJS), this method should work for the overwhelming majority of pinned insect material in collections worldwide, albeit with an expected larger fraction of reads removed due to mapping quality and read length.

CONCLUSION

In this paper we assess and optimize standard protocols for DNA extraction from pinned museum insect specimens and demonstrate that sufficient amounts of DNA for whole genome sequencing can reliably be extracted from small amounts of tissue, using either single legs (butterflies, destructively or non-destructively) or whole specimens (small wasps, non-destructively). We also find that this method is not particularly sensitive to the age of the specimens, with only minor differences between DNA extracted from specimens approximately 30 years old and specimens more than 100 years old. This method can thus be expected to work for the majority of insect material in museum collections, helping to unlock the full genomic potential of museum collections, and thus facilitating both temporal studies and studies of extinct or inaccessible taxa.

Based on our assessments and results from the hundreds of extractions performed with the selected protocol, we recommend using the Monarch PCR & DNA Cleanup Kit, a lysis temperature of 42°C and a lysis time of approximately 20 h (overnight). Generally, a non-destructive extraction provides enough DNA. However, for some species a destructive approach can substantially increase yield. If a destructive approach is superior with respect to yield, but not feasible due to restrictions on damage to specimens, a 2nd non-destructive extraction can increase overall DNA yield.

ADDENDUM. New England Biolabs discontinued the Monarch PCR & DNA Cleanup Kit in 2024 and replaced it with the Monarch Spin PCR & DNA Cleanup Kit. We have now performed 250+ extractions from single legs from museum specimens of Lepidoptera using the Monarch Spin Kit (see Supplement S4 for protocol). These specimens were 33–157 years old, and the yields of DNA were comparable to those achieved using the Monarch kit. Detailed data on these specimens are not included in this paper.

ACKNOWLEDGEMENTS. This study was greatly helped by the assistance of several people and institutions in facilitating access to collections: We would like to thank L. Kaila (Finnish

Museum of Natural History, Finland, LUOMUS), A. Teräs (Biological Collections of Åbo Akademi University & Zoological Museum, University of Turku, Finland), T. Kumpulainen (Tampere Museum of Natural History, Finland), T. Pape (Natural History Museum, Denmark), and M. Gates (Systematic Entomology Laboratory, USDA-ARS, National Museum of Natural History, USA). We would also like to thank N. Wahlberg for sharing his experience with the best method to extract DNA from old insect material and F. Patzold for answering questions regarding her extraction protocol using the Monarch Kit. We would like to thank M. Vestergård and A. Brandstrup for assistance in the lab. We are grateful for helpful input from our colleagues in the Centre for Ecological Genetics. M. Djernæs, A. Bras, T.J. Simonsen, M.R. Jensen, J.S. Bechsgaard, J.B. Pedersen, T. Bilde, and P.F. Thomsen gratefully acknowledge funding by the Novo Nordisk Challenge Programme grant number NNF20OC0060118.

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Received June 6, 2025; revised and accepted February 17, 2026
Published online February 26, 2026

Online supplementary files:

S1 (http://www.eje.cz/2026/008/S01.xlsx). Table S1. Collection dates, collection localities and NHMA (Natural History Museum Aarhus) database numbers for specimens used in protocol comparisons.

S2 (http://www.eje.cz/2026/008/S02.xlsx). Table S2. Sequencing metadata for individual specimens sequenced using sequenced using SCR library build and single end 50 bp sequencing.

S3 (http://www.eje.cz/2026/008/S03.pdf). Table S3. DNA extraction yields and percentage endogeneous DNA from previous studies extracting DNA from museum specimens of insects.

S4 (http://www.eje.cz/2026/008/S04.pdf). Protocols.