Comparing DNA isolation methods for forest trees: quality, plastic footprint, and time- efficiency

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Research Article

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Abstract

Background

Genetic and genomic studies are seeing an increase in sample sizes together with a wider range of species investigated in response to environmental change concerns. In turn, these changes may come with challenges including the time and difficulty to isolate nucleic acids (DNA or RNA), the sequencing cost and environmental impacts of the growing amount of plastic waste generated in the process. *Pseudotsuga menziesii* var. *menziesii* (Mirbel) Franco (PM), *Tsuga heterophylla* (Raf.) Sarg. (TH) and *Thuja plicata* Donn ex D.Don (TP) are conifer species found in diverse woodlands both as natives and naturalized exotics. Our study was carried out whilst investigating their genetics to understand their population structure and potential for adaptation.

Results

In the present study, we compared two different DNA isolation methods, i.e., spin-column DNeasy plant mini kit (QIAGEN), and temperature-driven enzymatic cocktail Plant DNA Extraction (MicroGEM). The quantity of recovered DNA and the quality of DNA were assessed along with the plastic footprint and time needed for three tree species. Both methods were optimised and proven to provide enough DNA for each studied species. The yield of DNA for each method depended on the species: QIAGEN showed higher yield in PM and TH, while TP recovered similar amount of DNA for both methods. The DNA quality was investigated using DNA barcoding techniques by confirming species identity and species discrimination. No difference was detected on the PCR amplification of the two barcoding loci, (*rbcL* and *trnH-psbA*), and the recovered sequences between DNA isolation methods. Measurement of the plastic use and the processing time per sample indicated that MicroGEM had a 52.64% lower plastic footprint and was 51.8% faster than QIAGEN.

Conclusions

QIAGEN gave higher yields in two of the species although both methods showed similar quality results across all species. However, MicroGEM was clearly advantageous to decrease the plastic footprint and improve the time efficiency. Overall, MicroGEM recovers sufficient and reliable DNA to perform common downstream analyses such as PCR and sequencing. Our findings illustrate the benefits of research and efforts towards developing more sustainable methods and techniques to reduce the environmental footprint of molecular analyses.

Background

Genetic and genomic analyses often benefit from a large sample set to reliably detect associations and identify patterns relevant to key biological questions [1] due to higher statistical power and accuracy [2]. However, larger sample sizes require more resources, such as time to collect and process the samples and the volume of consumables used in the laboratory [3]. Analyses commonly used include Genome-wide association studies (GWAS) [4], genomic selection (GS) and Genome environment association (GEA) to scan genomes for associations with complex traits of interest as in tree breeding programs [5] where the power to detect loci with small effects may be constrained by the sample size [6]. The minimum sample size for each genetic analysis varies depending on the research question, the genetic markers used, the biological system and the available resources. Environmental change [7] and its effects on natural habitats as well as in the distribution, population size and genetic diversity [7–11] increases the number of species at risk and in need to be studied [10]. Therefore, both the range and the size of studies involving molecular analyses are increasing [12].

Molecular studies on non-model tree species may face challenges including obtaining high-quality DNA and RNA, accessing wild populations, the complexity and size of their genome, and the limited available genomic resources [13]. These factors increase the complexity and the resource intensity of genomic studies in trees compared to studies of many other organisms [14]. The challenges of obtaining high-quality DNA and RNA from trees includes the difficulty to break down the cell walls to release the nucleic acids [15] and the inhibitory effects of secondary metabolites and polysaccharides on downstream applications such as PCR (polymerase chain reaction), sequencing and genotyping. Simple and robust protocols for isolation of high-quality nucleic acids from tree tissues that are suitable for downstream applications would help to overcome these challenges.

The main limitations when designing genomic experiments with large sample sizes include the time and difficulty to isolate nucleic acids (DNA or RNA), the sequencing cost and the environmental impact of plastic waste generated in the process. Sequencing platforms have reduced nucleic acids sequencing costs over the last two decades [16], making sequencing more accessible to researchers studying non-model species and feasible to study larger sample sizes. Some cost-effective pipelines, workflows and strategies have also been developed to reduce costs and process time [17]. Single-use plastic consumables are a significant source of waste in many scientific fields [18, 19], with an estimated use of 5.5 million tonnes per year worldwide [20]; therefore, although they are essential to perform genomic analyses, they have a large environmental impact [21]. Improved laboratory sustainability is encouraged by organizations and labels such as My Green Lab [22] and The Sustainable Laboratory Practices Working Group (SLPWG) [23] which promote green practices in the laboratory to reduce their plastic footprint. A different approach is to reuse specific plastic items, for example, Grenova Solutions [24] developed an on-bench Pipette Tip Cleaning Machine (TipNovus) that is easy to integrate into laboratory routines. Another solution is to choose or develop protocols that use less plastic without compromising the experiment's outcome [3, 25].

Several methods have been proposed to overcome the difficulties when isolating DNA from tree species. Broadly, there are five main types of DNA isolation systems including organic extraction methods which use organic solvents like phenol and chloroform [26, 27], solid-phase extraction methods that use solid matrices, such as silica to bind and purify the DNA [28], precipitation methods which use salts and ethanol to precipitate the DNA [29], enzymatic digestion methods that use individual or a combination of enzymes to digest the samples and release the DNA [30] and use of magnetic bead-based coated with an agent which binds with DNA and isolates it from the cellular suspension [31], or magnetic ionic liquids [32]. These methods are usually combined and modified depending on the experimenter's needs [17, 33].

In this study, we compared the performance of two different DNA isolation methods in three conifer forest tree species: *Pseudotsuga menziesii* var. *menziesii* (Mirbel) Franco (PM), *Tsuga heterophylla* (Raf.) Sarg. (TH) and *Thuja plicata* Donn ex D.Don (TP). They are all large evergreen coniferous trees native to western North America where they appear together in both mixed natural forests and plantations. These species are widely found in Europe and North America in woodlands which provide a variety of ecosystem services including timber production, carbon sequestration, biodiversity and habitat conservation, water regulation, recreation, and tourism [34, 35]. We tested the recovered DNA quantity, quality, plastic footprint and time of the methods were: i) Plant DNA Extraction (MicroGEM International PLC, 2019) (MicroGEM) which uses a temperature controlled enzymatic cocktail and ii) DNeasy plant mini kit (Qiagen USA, Valencia, CA) (QIAGEN), a silica column-based method widely used to isolate DNA from conifer trees. In this context, the aim of this study is to find an efficient DNA isolation method for forest trees by looking at the recovered DNA quality, the plastic waste generated, and the energy required to produce it and the total time needed to process the samples using both DNA isolation methods. We verified the quality of the DNA recovered by use of PCR with gene specific primers followed by DNA sequencing.

Results

Improvement of DNA isolation methods and comparison of species

We isolated the DNA from four samples in each of the three study species by using MicroGEM and QIAGEN original and improved protocols (see methods for details). The improvements to DNA isolation methods increased the DNA recovered both for the MicroGEM and QIAGEN methods in TP, PM and TH (Fig. 1). The data suggest that DNA yields were generally higher with QIAGEN, except for TP where the results were similar across methods (Fig. 1, B and D). However, statistical comparison of modified methods and species (Fig. 1, B and D) showed significant effects of species and the interaction of species and methods but not between methods (Table 2). Overall, the modified procedures yielded more than 1 µg across all the species starting from a minimum of 10 mg of dried tissue (except of TH4 extracted with MicroGEM, Table 1), which is sufficient for many downstream analyses. Yields above 10 µg were obtained in PM and most of TH but only for QIAGEN method (Table 1). The samples extracted with the modified protocols of both DNA isolation methods will be used for the subsequent analyses.

Table 1
DNA yield across species with improved methods.

Sample ID	Conc.(ng/µl) MicroGEM	Yield (µg) MicroGEM	Conc.(ng/µl) QIAGEN	Yield (µg) QIAGEN
TH1	17.8	1.424	72.3	7.230
TH2	21.2	1.484	133	13.300
TH3	19.3	1.544	118	11.800
TH4	15.1	0.906	93.7	9.370
TP1	78	7.020	51.1	5.110
TP2	75	6.375	65	6.500
TP3	53.2	4.256	52.8	5.280
TP4	70.8	5.664	54.2	5.420
PM1	65.8	4.606	119	11.900
PM2	41.9	2.933	106	10.600
PM3	16.8	1.176	132	13.200
PM4	44.6	3.122	101	10.100

Table 2
Anova table of factors affecting DNA yield recovered including methods, species and their interaction. Sum Sq: Sum of squares, Df: Degrees of freedom, Pr: Probability. '***' corresponds to a P. value of 0.001.

Anova Table (Type III tests)	Sum Sq	Df	F value	Pr (> F)	Significance
(Intercept)	124434025.00	1	57.63	5.13E-07	***
Method	126253.13	1	0.06	8.12E-01	
Species	78713516.67	2	18.23	4.71E-05	***
Method:Species	109300794.75	2	25.31	5.88E-06	***
Residuals	38865031.50	18			

DNA recovered quality

The quality of the DNA recovered from each method was analysed with standard DNA Barcoding techniques with commonly used gene-specific primers, the large subunit of the ribulose-bisphosphate carboxylase gene (*rbcL*) and the plastid *trnH-psbA* intergenic spacer. Major amplicons of the expected size (Table 8) were detected in all the samples tested for both barcodes *rbcL* and *trnH-psbA* (Table 3, Fig. 2). Other minor bands appeared in the *rbcL* PCR products as displayed in the gel images while none were detected in the *trnH-psbA* gel (Fig. 2, Table 3). There were no difference between DNA isolation methods in *trnH-psbA* PCR products, but samples extracted with MicroGEM show minor bands in 75% of the *rbcL* products compared to only 33.3% of QIAGEN samples (Fig. 2, A and C).

Every *trnH-psbA* sequence retrieved was of the expected length amplicon size while 25% of the *rbcL* sequences were unreliable (Table 3). When looking at the differences between the DNA isolation methods, one QIAGEN sample and two MicroGEM samples could not be sequenced satisfactorily for *rbcL*.

Table 3
Summary of PCR and Sequencing results for each DNA isolation method per species and barcode marker.

		PCR					Sequencing		
			rbcL		*trnH-psbA*			*rbcL*	*trnH-psbA*
DNA isolation Method	Species	N	Mayor amplicon detected	Minor bands detected	Mayor amplicon detected	Minor bands detected	N	Expected seq. length	Expected seq. length
QIAGEN	TP	4	100%	50%	100%	0%	2	100%	100%
	PM		100%	50%	100%	0%		50%	100%
	TH		100%	0%	100%	0%		100%	100%
MicroGEM	TP		100%	75%	100%	0%		50%	100%
	PM		100%	100%	100%	0%		50%	100%
	TH		100%	50%	100%	0%		100%	100%

We submitted the *rbcL* and *trnH-psbA* recovered sequences to BOLD and GENBANK, respectively to verify the species identities of the tested samples. All sequences returned the correct species identification, except for *rbcL* in samples whose sequence length was shorter than the expected size (Table 4). Two of the problematic sequences were from samples isolated with MicroGEM (TP and PM) and one from QIAGEN (PM) (Table 4).

Table 4
Species identification using GENEBANK and BOLD datasets for both barcodes.
N: Number of samples.

DNA isolation method	Species	N	BOLD *rbcL*	GENBANK *trnH-psbA*
QIAGEN	TP	2	2	2
	PM	2	1	2
	TH	2	2	2
MicroGEM	TP	2	1	2
	PM	2	1	2
	TH	2	2	2

The sequences obtained of the *trnH-psbA* amplicons were clustered using USEARCH v.11 to assess the species discrimination efficacy of both methods. A clustering was determined for the three species and the samples were classified in species-specific clusters with a level of similarity above 99% in every case, without any difference between DNA isolation methods.

DNA obtained on large populations

Populations with larger numbers of individuals have been processed in two of the species described above with both methods in a parallel project (Table 5). The QIAGEN method gave higher mean yields for TP than PM with the latter also being more consistent as show by lower levels of variation (CV) (Table 5). The yield recovered from TP samples was lower and slightly variable when using MicroGEM than QIAGEN (Table 5).

Table 5
Summary table of the DNA yield recovered in a larger project when using both DNA isolation methods. CV: Coefficient variation, N: Number of samples.

Method	Species	Yield Mean (µg)	Yield CV	N
QIAGEN	TP	9.603	59.24%	79
	PM	7.766	40.37%	1146
MicroGEM	TP	3.312	66.09%	472

Plastic footprint and time needed for DNA isolations

The plastic consumed was determined for all steps used in each method, including the clean-up step using AMPureXP beads needed after the DNA isolation using MicroGEM (Fig. 3). To extract one sample, 12.51g of plastic was used with QIAGEN and 5.92g with MicroGEM + AMPureXP beads from both tube and tip items (Fig. 3). Overall, MicroGEM required 52.64% less plastic than QIAGEN to isolate DNA, per sample. The plastic footprint was determined for both CO2 emissions and energy consumption to produce the required plastic to process a single sample. The data showed that the plastic footprint was at least 50% lower with MicroGem (Table 6).

Table 6
Plastic footprint of the DNA isolation methods to process one sample.

	Carbon emissions (Kg CO2)	Energy used (Mj)
QIAGEN	0.04255	1.07498
MicroGEM	0.02011	0.50809

We also measured the time required to isolate DNA with each method by determining the time needed to process 24 samples and calculated for a single sample (see methods for details). Overall, the time required to extract a single sample using MicroGEM was 51.8% less than QIAGEN (Table 7). If we only include the hands-on time needed to purify the DNA of one sample by using both methods, then MicroGEM needed 34.6% less time than QIAGEN.

Table 7
Time required to process one sample by using each of the DNA isolation methods.

	Total (min)	Total hands-on (min)
QIAGEN	119.4	5.4
MicroGEM	57.5	3.5

The plastic consumed to isolate 1146 PM and 79 TP sample from the population project described above (see Table 5) by using the DNA isolation QIAGEN method was 15.3 kg, which represented 52.06 kg of CO2 emitted, and 1310 Mj of energy required to produce the plastic used. If MicroGEM was used to isolate the DNA of this project rather than QIAGEN, 8.1 kg less plastic would have been utilised, 27.44 kg of CO2 emission would have been avoided and 686 Mj of energy would have been saved. Based on an 8-hour working day, 25.9 days were needed to complete the 1225 isolations using QIAGEN while only 14.7 days would have been needed to isolate the same samples if using MicroGEM.

Discussion

The central question posed in this study was if a rapid and plastic-efficient DNA isolation method will recover reliable DNA similarly to commonly used kit. We compared the DNA yield, DNA quality and efficiency on four samples of three different forest tree species and considered outputs of a separate study in a larger population.

We presented data on the use of different methods to isolate nucleic acids from forest trees including method optimisations to the basic protocols to isolate the DNA compared to the original methods. No significant difference in DNA yield recovered was found between the two methods when using the improved protocols, indicating that either method will deliver sufficient DNA yield for standard molecular analyses. However, significant differences were found among species, which suggested the need for specific optimisation of laboratories protocols when looking at various species. The significant difference found in the interaction between methods and species suggest that PM and TH recovered more DNA when using QIAGEN, but TP recovered similar amounts in both cases. Several studies looked at the differences in amount of recovered DNA quality and time efficiency of different DNA isolation methods in plant species [36]. Bashalkhanov & Rajora, (2008) tested several DNA extraction systems suitable for conifers obtaining a mean DNA yield of more than 10 µg when using the QIAGEN DNeasy kit, which aligns with our DNA yield results. They also concluded that the QIAGEN DNeasy kit is not a preferred method when dealing with large numbers of samples due to the long time that is required to complete the isolation. Our initial MicroGEM DNA concentration results were similar to previous research such as described by Ryan et al. [38], which showed a very low recovered DNA concentration (less than 3 ng/µl) when using MIGROGEM on different plant tissues. After optimisation, we substantially increased the concentration to over 15 ng/µl and up to 78 ng/µl using MicroGEM. These results confirmed the utility of optimising protocols to obtain sufficient DNA to perform reliable downstream analyses.

The quality of the DNA was analysed with standard DNA Barcoding techniques with gene-specific primers as [[39] for *rbcL* and *trnH-psbA*, which are commonly used to confirm the species identity and power of discrimination by PCR amplification and sequencing [40, 41]. For *trnH-psbA*, both DNA isolation methods delivered accurate size amplicons and no minor bands. When examining the amplified fragments from the *rbcL* marker, more minor bands were found on those samples extracted by MIGROGEM, making them less suitable in subsequent analysis. All sequences retrieved from *trnH-psbA* were of the expected length, assuring a better performance on subsequent analyses. The sequences from *rbcL* and *trnH-psbA* markers were submitted to BOLD and GenBank, respectively, to confirm sample identities. DNA samples isolated with both methods that yielded sequences of the expected length were correctly identified. In the species discrimination analysis with the *trnH-psbA*, all samples from the same species clustered together, without any differences

between the DNA isolation method. Several studies have validated these two loci for efficient DNA barcoding in plants [42], and specifically conifers [39]. In their study, Armenise et al. [40] results for PM using these same two markers recovered the expected fragment size after performing the PCR as well as the expected sequence length. Our sequence lengths for PM are consistent with Armenise et al. [40], retrieving 710 bp with *rbcL* and 565 bp in *trnH-psbA*, showing that the amplifications performed consistently in both cases. Their findings suggest that combining these two markers may be preferable to perform DNA Barcoding in conifers due to their PCR uniformity and sufficient sequence quality while showing enough variation to perform species identity analyses at the genus level. In contrast, our results suggest that using *trnH-psbA* alone retrieves sufficient evidence to identify and differentiate species from different genus.

To assess the plastic and time efficiency of each DNA isolation method, we reported the plastic footprint and the time needed to process one sample. The results of this study indicate that a temperature-driven enzymatic cocktail isolation system reduces plastic footprint by 52.64% compared to a commonly used silica-based nucleic acid isolation method. This enzymatic method also appears to reduce the average time needed to process a sample by 51.8%. When looking at the plastic footprint, we found that there is limited research on the plastic footprint that compares plastic consumption of different isolation nucleic acids methods. Marengo et al. [43] developed a DNA isolation method for plant species where they reduced the sample processing time while maintaining the quality of the DNA recovered. The authors also discussed the reduction of plastic waste achieved although this was not quantified. The lack of previous research on this matter prevents a direct comparison with our DNA isolation plastic footprint results. Nonetheless, there is an increase of studies looking at how to reduce general plastic use in laboratories [3, 19] and proposing protocols optimised to decrease the amount of plastic use [25]. Alves et al. [3] developed a 7-week study measuring the plastic used in a molecular laboratory after promoting very specific behavioural and protocol changes to reduce the use of plastic items. They were able to achieve a reduction of more than 10 kg of plastic per week on average, from an initial of 24 kg. The improved protocol for GBS library preparation proposed by Torkamaneh et al. [25] reduces both the time and plastic needed by 75% and 89%, respectively, compared to standard methods. These findings, together with our results, confirm the potential to apply sustainable measures such as using alternative materials, reusing items when possible or developing protocols which use less plastic without compromising the experiment's outcome.

Our study comparing different methods shows the potential to significantly impact on the plastic consumption and efficiency of DNA isolation in coniferous forest tree species. Our findings suggest that MicroGEM is a highly suitable method as it provided sufficient DNA yield with good quality while producing the least amount of plastic waste and being the most time efficient. Our study also shows that selecting the most suitable method will depend on the specific requirements of the project, the species studied and the resources available. We believe that our results will encourage researchers to select DNA isolation methods based on sustainable laboratory practices, although further research is needed to explore the performance of this method on a broader range of species and molecular analyses.

Conclusion

In conclusion, the main result of this study is that this rapid and plastic-efficient method, Plant DNA Extraction, MicroGEM, recovers sufficient and reliable DNA to perform common downstream analyses such as PCR and sequencing, and performs as well as a commonly used spin-column kit, DNeasy plant mini kit, QIAGEN. Our study highlights the merit of efforts towards developing more sustainable and efficient laboratory practices in the field of molecular biology. A reduction of plastic waste in molecular laboratories deserves further research for the development of techniques and protocols that use less single-use plastic items.

Methods

The methodology and sequence of major steps implemented across this study (Fig. 4), included: 1) plant material collection, 2) DNA isolation, 3) DNA yield quantification and 4) DNA quality assessment by using DNA barcoding for species identification and species discrimination by performing PCR and followed by DNA sequencing.

Plant material

The study species are the conifers *Thuja plicata* (TP), *Pseudotsuga menziesii* (PM) and *Tsuga heterophylla* (TH). They are all part of the Pinales order; TP belongs to the Cupressaceae, whilst PM and TH belong to the Pinaceae. Four samples per species were retrieved from different planted stands in England using an arborist slingshot following a modified method based on Youngentob et al. [44]. The leaves collected were dried by storing them in silica gel beads after removing them from the tree and until processing for DNA isolation.

DNA isolation procedures and improvements

We isolated the DNA using 20 mg of leaf tissue from the 12 samples by using the manufacturer's instructions for the DNeasy plant mini kit (Qiagen USA, Valencia, CA) (QIAGEN), we also modified the procedure to enhance the quality and yield of the DNA recovered, by adding 20 µl of Protease K in the lysate step and increasing the incubation time to a minimum of an hour at 55° C. [45].

We processed the same 12 samples using the DNA isolation method Plant DNA Extraction [46] (MicroGEM) following the manufacturer's original instructions. First, 10 mg of leaf tissue is mixed with the lysis buffer and using a mechanical homogenizer to break the cells, second, the lysate is transferred to a PDQeX DNA-binding cartridge and third, the enzymatic cocktail is added to the cartridge, which is inserted into the PDQeX device which through the combination of changes in temperature and the physical design of the cartridge performs a chemical process that releases the DNA into the recovery tubes. We also modified this procedure to optimise the DNA yield as follows: 1) The tissue was disrupted alone and added with a micro scoop to the pre-sample mix which included the lysate buffer and the enzymatic cocktail in individual tubes, 2) the sample mix was pipette into the cartridges, which were inserted into the PDQex device with a modified plant programme with an incubation time increased from 5 min to 15 min and extraction time from 5 min to 10 min [47].

After recovering the DNA we performed a clean-up step by using the AMPpureXP beads [48], which use magnetic particles that bind the DNA to allow the clean-up. A concentration of 0.8X of AMPureXP beads (Beckman Coulter™, Brea, CA, USA) was used for the size selection needed to retrieve the isolated DNA fragments (recommended by NewEngland Biolabs).

DNA recovery

The DNA yield recovered from all samples was quantified using Qubit® Fluorometer v.4.0 (Invitrogen™, Thermo Fisher Scientific, Cleveland, OH, USA) (Fig. 1). The samples extracted by the modified protocols were used in this study (for details, see Table 1). DNA quality of these samples was visualized by electrophoresis in 0.8% Agarose gels (TAE Buffer), which confirmed the DNA presence (not shown). We performed an ANOVA test (error type iii) using the R package 'ape' [49] to analyse the differences in DNA yield recovered between methods and species. To visualise the data, we used the R package 'ggplot2' [50].

DNA analyses

The quality of the DNA was analysed by PCR amplification with gene-specific primers: the large subunit of the ribulose-bisphosphate carboxylase gene (*rbcL*) and the plastid *trnH-psbA* intergenic spacer, and sequencing on each sample. We adjusted the DNA concentrations to 20ng/µl, when possible (Table 1), for PCR amplification of *rbcL* and *trnH-psbA*

barcoding loci (Table 8). We used Q5® High-Fidelity 2X Master Mix DNA Polymerase (NewEngland Biolabs®, Ipswich, MA, USA) and added 100 ng of template DNA and 0.5 µM of each primer into a 25 µl final reaction following the manufacturer's instructions. The thermocycler conditions were 98°C for 20 seconds, followed by 40 cycles starting at 98°C for 10 seconds, TA (*rbcL*: 54°C and *trnH-psbA*: 63°C, Table 8) for 30 seconds and 72°C for 30 seconds, with a final extension at 72°C for 2 minutes. The amplified regions were visualized by electrophoresis in a 1.8% Agarose gel with SYBR safe (Invitrogen™, Thermo Fisher Scientific, Cleveland, OH, USA) as stain.

Table 8
Locus and primer details for PCR. TA: Annealing temperature used.

Marker name	Primer's sequence	TA (°C)	Expected product size (bp)	Source
trnH-psbA	CGCGCATGGTGGATTCACAATCC	63	500	[51]
	GTTATGCATGAACGTAATGCTC			
rbcL	ATGTCACCACAAACAGAAAC	54	750	[52]
	TCGCATGTACCTGCAGTAGC			

PCR products of two samples per species for each of the extraction methods were analysed by Sanger sequencing (Source Biosciences). Pre-cleaning and post-quality checks were performed by the sequencing service facilitator. Retrieved sequences were visualized and the electropherograms manually checked by using SnapGene software [53]. We exported the fasta files from the ab1 files which were trimmed with Trimmomatic [54] and aligned with MUSCLE [55].

We submitted the *rbcL* trimmed sequences to The Barcode of Life Data system v4 (BOLD) [56] and the *trnH-psbA* to the nucleotide dataset from GenBank [57]. BOLD enables a species-level identification of plant taxa by submitting queries of *MatK* and *rbcL* sequences to be searched against their reference library through their sequence threshold identification system. We verified the species discrimination efficacy of the *trnH-psbA* barcode by clustering the aligned sequences together by similarity using the software USEARCH v.11 [58]. The UCLUST algorithm divides a set of sequences into clusters providing a high-throughput species-level discrimination. The minimum similarity level was set to 99%.

Plastic footprint and time needed for DNA isolations

The plastic used to isolate DNA with both modified methods was calculated for a single reaction by weighing every plastic item with a precision balance (Table 9) not including the packaging.

Table 9
Weight of plastic items required, and number of items needed per DNA isolation method to process one sample. N: number of items.

Plastic item	Weight (g)	QIAGEN (N)	MicroGEM (N)
Cartridge	1.183	0	1
2ml tube	1.089	1	1
1.5mL tube	0.94	1	0
2mL no-Lid tube	0.932	3	0
Column	0.734	2	0
0.5mL tube	0.461	0	1
0.2mL tube	0.153	0	1
1000ul tip	1.033	5.13	0.17
200ul tip	0.315	2.04	0.08
20ul tip	0.219	0.04	0.00
10ul tip	0.275	1	0.00
cut tip	0.311	0.00	1.00
Total		15.21	5.25

The carbon dioxide emitted to produce 1 kg of polypropylene plastic commonly used to make laboratory supplies has been discussed by Harding et al. [57] and 3.4 kg CO2 is generally accepted amount. The total energy required to produce 1 kg of plastic from the extraction of raw materials to the final manufactured product is 85.9 MJ [59]. Following these data, we calculated the kg of CO2 emissions and total energy required to process one sample with each DNA isolation method based on the amount of plastic needed in each case.

The time required to isolate DNA by using both modified methods was timed for each step in the procedures when processing 24 samples and calculated for a single reaction. Incubation time and centrifuge steps time are fixed and remain the same for either 1 or 24 samples. We divided the hands-on time by 24 samples and added it to the measured fixed time.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LG drafted the manuscript and both LG and JM revised the main manuscript and LG conceived and planned the experiments, performed laboratory and statistical analyses and prepared the figures. JM supervised the project.

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References

1. Fumagalli M. Assessing the Effect of Sequencing Depth and Sample Size in Population Genetics Inferences. PLoS ONE. 2013;8:e79667.
2. Hong EP, Park JW. Sample Size and Statistical Power Calculation in Genetic Association Studies. Genomics Inf. 2012;10:117.
3. Alves J, Sargison FA, Stawarz H, Fox WB, Huete SG, Hassan A et al. A case report: insights into reducing plastic waste in a microbiology laboratory. Access Microbiol. 2021;3.
4. Visscher PM, Wray NR, Zhang Q, Sklar P, McCarthy MI, Brown MA, et al. 10 Years of GWAS Discovery: Biology, Function, and Translation. Am J Hum Genet. 2017;101:5–22.
5. Müller BSF, de Almeida Filho JE, Lima BM, Garcia CC, Missiaggia A, Aguiar AM, et al. Independent and Joint-GWAS for growth traits in Eucalyptus by assembling genome-wide data for 3373 individuals across four breeding populations. New Phytol. 2019;221:818–33.
6. Tan B, Ingvarsson PK. Integrating genome-wide association mapping of additive and dominance genetic effects to improve genomic prediction accuracy in Eucalyptus. Plant Genome. 2022;15:e20208.
7. Brown SC, Wigley TML, Otto-Bliesner BL, Rahbek C, Fordham DA. Persistent Quaternary climate refugia are hospices for biodiversity in the Anthropocene. Nat Clim Change 2020. 2020;10:3.
8. Pauls SU, Nowak C, Bálint M, Pfenninger M. The impact of global climate change on genetic diversity within populations and species. Mol Ecol. 2013;22:925–46.
9. Heuertz M, Carvalho SB, Galindo J, Rinkevich B, Robakowski P, Aavik T et al. The application gap: Genomics for biodiversity and ecosystem service management. Biol Conserv. 2023;278.
10. Harley CDG. Climate change, keystone predation, and biodiversity loss. Science (1979). 2011;334:1124–7.

11. Garden JG, O'Donnell T, Catterall CP. Changing habitat areas and static reserves: challenges to species protection under climate change. Landsc Ecol. 2015;30:1959–73.
12. Alberto FJ, Aitken SN, Alía R, González-Martínez SC, Hänninen H, Kremer A, et al. Potential for evolutionary responses to climate change – evidence from tree populations. Glob Chang Biol. 2013;19:1645.
13. Holliday JA, Aitken SN, Cooke JEK, Fady B, Gonz Alez-Martinez SC, Heuertz M, et al. Advances in ecological genomics in forest trees and applications to genetic resources conservation and breeding. Mol Ecol. 2017;26:706–17.
14. Abril N, Gion JM, Kerner R, Müller-Starck G, Cerrillo RMN, Plomion C, et al. Proteomics research on forest trees, the most recalcitrant and orphan plant species. Phytochemistry. 2011;72:1219–42.
15. Rezadoost MH, Kordrostami M, Kumleh HH. An efficient protocol for isolation of inhibitor-free nucleic acids even from recalcitrant plants. 3 Biotech. 2016;6:1–7.
16. Levy SE, Boone BE. Next-Generation Sequencing Strategies. Cold Spring Harb Perspect Med. 2019;9.
17. FU Z yu SONGJ, cheng, Jameson PE. A rapid and cost effective protocol for plant genomic DNA isolation using regenerated silica columns in combination with CTAB extraction. J Integr Agric. 2017;16:1682–8.
18. Sawyer A. The unsustainable lab. Biotechniques. 2019;66:5–7.
19. Howes L. Can Laboratories Move Away from Single-Use Plastic? ACS Cent Sci. 2019;5:1904–6.
20. Urbina MA, Watts AJR, Reardon EE. Labs should cut plastic waste too. Nature. 2015;528:479–9.
21. Di Paolo L, Abbate S, Celani E, Di Battista D, Candeloro G. Carbon Footprint of Single-Use Plastic Items and Their Substitution. Sustainability 2022, Vol 14, Page 16563. 2022;14:16563.
22. My Green Lab. https://www.mygreenlab.org/. Accessed 7 May 2023.
23. The Sustainable Laboratory Practices Working Group (SLPWG). https://nems.nih.gov/greening-tools/Pages/Sustainable-Labs.aspx. Accessed 7 May 2023.
24. Grenova Solutions. https://grenovasolutions.com/. Accessed 7 May 2023.
25. Torkamaneh D, Boyle B, St-Cyr J, Légaré G, Pomerleau S, Belzile F. NanoGBS: A Miniaturized Procedure for GBS Library Preparation. Front Genet. 2020;11.
26. Green MR, Sambrook J. Isolation of High-Molecular-Weight DNA Using Organic Solvents. Cold Spring Harb Protoc. 2017;2017:pdb.prot093450.
27. Doyle JJ. Isolation of plant DNA from fresh tissue. Focus. 1990;12.
28. Vega-Vela NE, Chacón Sánchez MI. Isolation of high-quality DNA in 16 aromatic and medicinal Colombian species using silica-based extraction columns. Agron Colomb. 2011;29:349–57.
29. Green MR, Sambrook J. Precipitation of DNA with ethanol. Cold Spring Harb Protoc. 2016;2016:1116–20.
30. Manen J-F, Sinitsyna O, Aeschbach L, Markov AV, Sinitsyn A. A fully automatable enzymatic method for DNA extraction from plant tissues. BMC Plant Biol. 2005;5:23.
31. Azimi SM, Nixon G, Ahern J, Balachandran W. A magnetic bead-based DNA extraction and purification microfluidic device. Microfluid Nanofluidics. 2011;11:157–65.
32. Emaus MN, Cagliero C, Gostel MR, Johnson G, Anderson JL. Simple and efficient isolation of plant genomic DNA using magnetic ionic liquids. Plant Methods. 2022;18:1–14.
33. Li J, Wang S, Yu J, Wang L, Zhou S. A Modified CTAB Protocol for Plant DNA Extraction. Chin Bull Bot. 2013;48:72.
34. Mina M, Bugmann H, Cordonnier T, Irauschek F, Klopcic M, Pardos M, et al. Future ecosystem services from European mountain forests under climate change. J Appl Ecol. 2017;54:389–401.
35. Bastien J-C. Douglas-fir biomass production and carbon sequestration. 4.2. Douglas-fir – an option for Europe. In: European Forest Institute, What Science can tell us; 2019.

36. Pipan B, Zupančič M, Blatnik E, Dolničar P, Meglič V. Comparison of six genomic DNA extraction methods for molecular downstream applications of apple tree (*Malus X domestica*). Cogent Food Agric. 2018;4:1540094.
37. Bashalkhanov S, Rajora OP, Protocol. A high-throughput DNA extraction system suitable for conifers. Plant Methods 2008. 2008;4(1):4:1–6.
38. Ryan AL, O'Hern CP, Elkins KM. Evaluation of Two New Methods for DNA Extraction of "Legal High" Plant Species. J Forensic Sci. 2020;65:1704–8.
39. Armenise L, Simeone MC, Piredda R, Schirone B. Validation of DNA barcoding as an efficient tool for taxon identification and detection of species diversity in Italian conifers. Eur J For Res. 2012;131:1337–53.
40. Hollingsworth PM, Forrest LL, Spouge JL, Hajibabaei M, Ratnasingham S, van der Bank M, et al. A DNA barcode for land plants. Proc Natl Acad Sci U S A. 2009;106:12794–7.
41. Loera-Sánchez M, Studer B, Kölliker R. DNA barcode trnH-psbA is a promising candidate for efficient identification of forage legumes and grasses. BMC Res Notes. 2020;13:1–6.
42. Kress WJ, Erickson DL. A Two-Locus Global DNA Barcode for Land Plants: The Coding rbcL Gene Complements the Non-Coding trnH-psbA Spacer Region. PLoS ONE. 2007;2:e508.
43. Marengo A, Cagliero C, Sgorbini B, Anderson JL, Emaus MN, Bicchi C, et al. Development of an innovative and sustainable one-step method for rapid plant DNA isolation for targeted PCR using magnetic ionic liquids. Plant Methods. 2019;15:1–11.
44. Youngentob KN, Zdenek C, van Gorsel E. A simple and effective method to collect leaves and seeds from tall trees. Methods Ecol Evol. 2016;7:1119–23.
45. Guillardín L. QIAGEN DNeasy Plant Mini Kit DNA extraction modified protocol - Douglas fir. website. https://lauguillardin.github.io/2022-07-12-microGEM-DNAextraction_Thujaplicata/. Accessed 27 Mar 2023.
46. MicroGEM International PLC, Microgem. Nucleic Acid Extraction: Extremely Fast, Extremely Simple, Extremely Effective. Genetic Eng Biotechnol News. 2019;39:15–S15.
47. Guillardin L, microGEM. DNA extraction modified protocol - Western redcedar. website. https://lauguillardin.github.io/2022-07-12-microGEM-DNAextraction_Thujaplicata/. Accessed 27 Mar 2023.
48. Deangelis MM, Wang DG, Hawkins TL. Solid-phase reversible immobilization for the isolation of PCR products. Nucleic Acids Res. 1995;23:4742.
49. Paradis E, Schliep K. ape 5.0: an environment for modern phylogenetics and evolutionary analyses in R. Bioinformatics. 2019;35:526–8.
50. Wickham H. ggplot2: Elegant Graphics for Data Analysis. New York, NY: Springer New York; 2009.
51. Kress WJ, Wurdack KJ, Zimmer EA, Weigt LA, Janzen DH. Use of DNA barcodes to identify flowering plants. Proc Natl Acad Sci U S A. 2005;102:8369–74.
52. SnapGene, Software. (https://www.snapgene.com/).
53. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30:2114–20.
54. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32:1792–7.
55. Ratnasingham S, Hebert PDN. bold: The Barcode of Life Data System (http://www.barcodinglife.org). Mol Ecol Notes. 2007;7:355–64.
56. Clark K, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank Nucleic Acids Res. 2016;44:D67–72.
57. Edgar RC, Bateman A. Search and clustering orders of magnitude faster than BLAST. Bioinf Appl NOTE. 2010;26:2460–1.

58. Harding KG, Dennis JS, von Blottnitz H, Harrison STL. Environmental analysis of plastic production processes: Comparing petroleum-based polypropylene and polyethylene with biologically-based poly-β-hydroxybutyric acid using life cycle analysis. J Biotechnol. 2007;130:57–66.

Figures

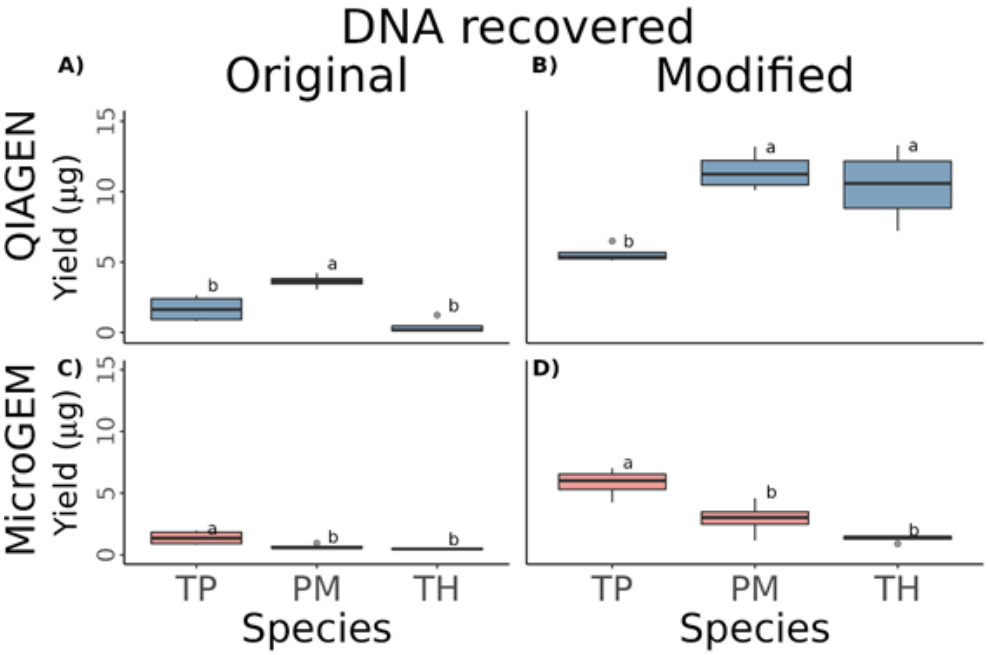

Figure 1

DNA yield recovered by A) using QIAGEN original protocol, B) QIAGEN modified protocol, C) MicroGEM original protocol and D) modified MicroGEM protocol. TP: Thuja plicata, PM: Pseudotsuga menziesii and TH: Tsuga heterophylla. Letters represent significant differences between species for each of the protocols tested.

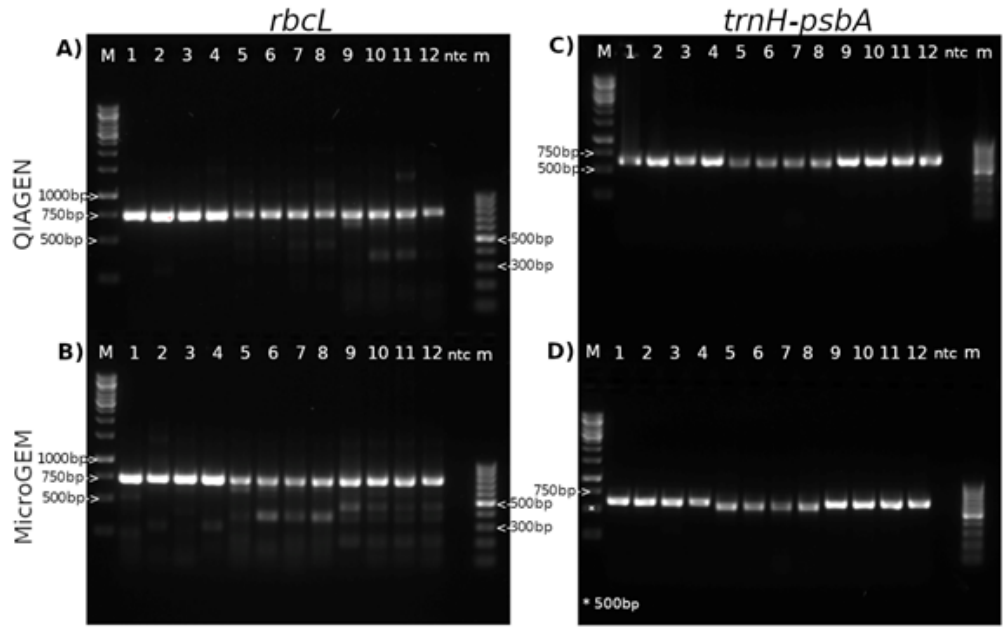

Figure 2

rbcL PCR product for A) QIAGEN B) MicroGEM samples and *trnH-psbA*for C) QIAGEN and D) MicroGEM samples. Sample names: M: 1Kb molecular-weight size marker, 1:TH1, 2:TH2, 3:TH3, 4:TH4, 5:TP1, 6:TP2, 7:TP3, 8:TP4, 9:PM1, 10:PM2, 11:PM3, 12:PM4, ntc: non-template control, m: 100bp molecular size marker.

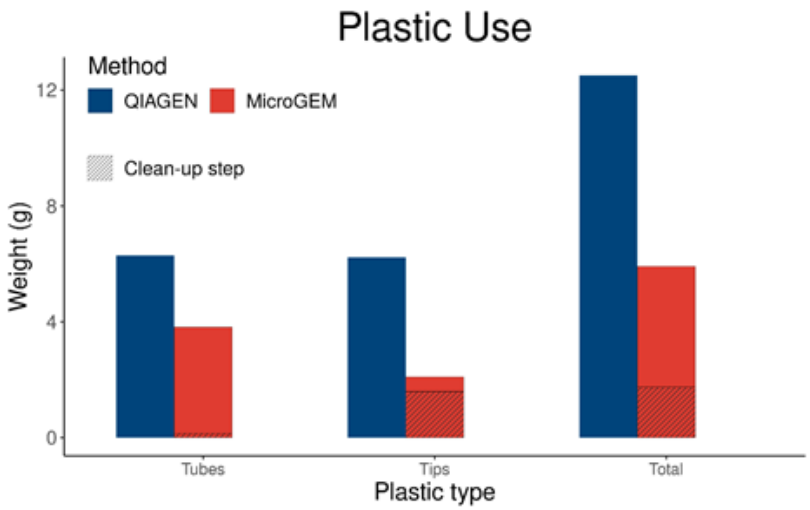

Figure 3

Total plastic required to process one sample by QIAGEN (blue) and MicroGEM (red). The clean-up step plastic use (MicroGEM only) hatched.

Diagram of the methodology followed

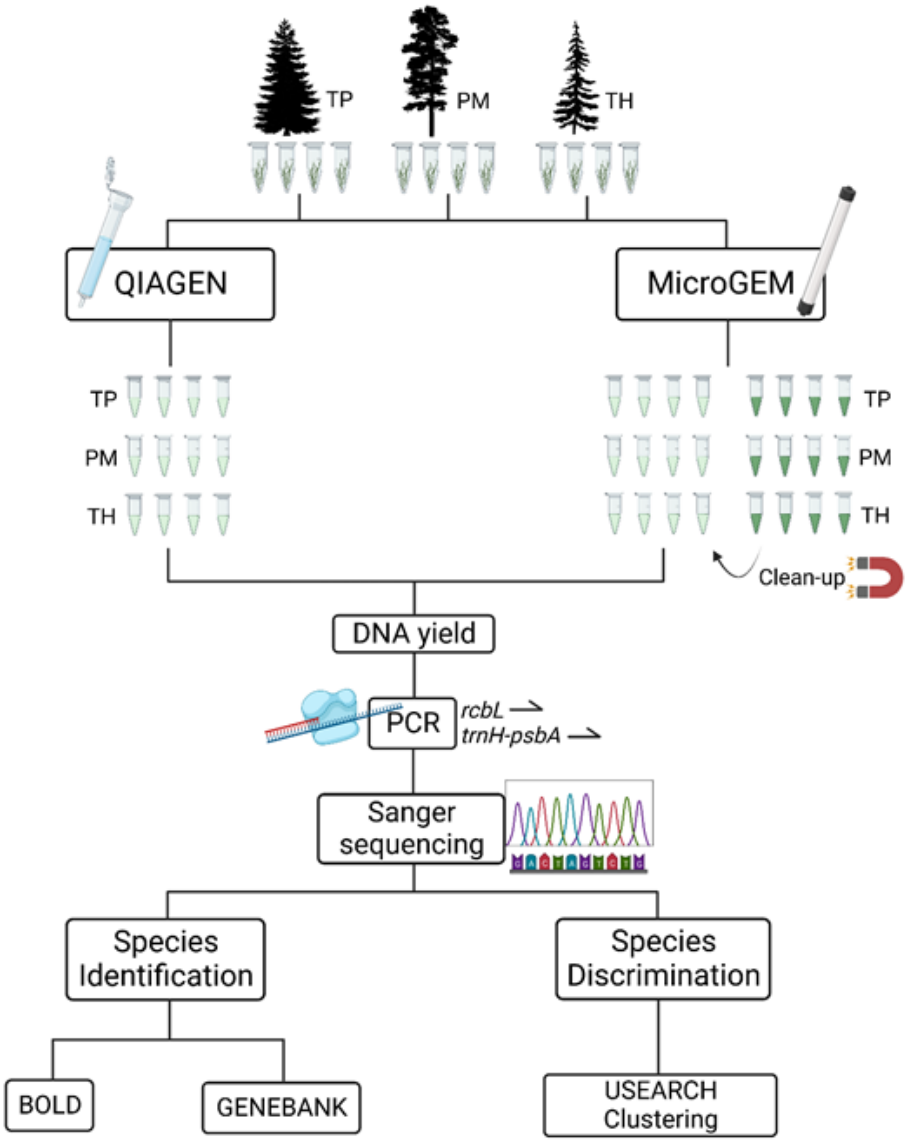

Figure 4

Diagram of the methodology followed.