



Optimization of DNA extraction from fresh and dried *Pinus brutia* needles using different homogenization methods: An approach for forensic botanical applications

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Taze ve kurutulmuş *Pinus brutia* iğne yapraklarından DNA ekstraksiyonunun farklı homojenizasyon yöntemleriyle optimizasyonu: Adli botanik uygulamaları için bir yaklaşım

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Abstract: Obtaining sufficient DNA from plant tissues is essential for molecular taxonomy, population genetics, conservation biology, and forensic botany. However, DNA extraction from conifer species remains challenging because their tissues contain high concentrations of polysaccharides, polyphenols, terpenoids, and resin compounds that may interfere with DNA isolation and downstream molecular analyses. This study aimed to evaluate the effects of sample condition and homogenization method on DNA concentration obtained from fresh and dried needles of *Pinus brutia*, one of the most widespread conifer species in Türkiye. Needle samples were collected from 24 individual trees in Çanakkale, Türkiye. Samples from 12 trees were processed fresh, whereas samples from the remaining 12 trees were air-dried for 20 days. Within each sample condition, samples were processed using two homogenization methods: mortar grinding followed by TissueLyser homogenization and blender homogenization. Genomic DNA was extracted using the i-genomic Plant DNA Extraction Mini Kit and quantified using a Qubit™ 4 Fluorometer with the dsDNA High Sensitivity Assay. Data were analyzed using two-way analysis of variance (ANOVA). Fresh samples showed higher DNA concentrations than dried samples, and mortar grinding followed by TissueLyser homogenization resulted in higher DNA concentrations than blender homogenization. Two-way ANOVA demonstrated significant main effects of sample condition and homogenization method on DNA concentration, as well as a significant interaction between the two factors. These findings indicate that both sample condition and homogenization method influence DNA concentration obtained from *P. brutia* needles and highlight the importance of sample-specific optimization of DNA extraction procedures for molecular plant research and forensic botanical applications.

Key words: Molecular plant biology, forensic botany, DNA extraction, homogenization, *Pinus brutia*

Özet: Bitki dokularından yeterli miktarda DNA elde edilmesi; moleküler taksonomi, popülasyon genetiği, koruma biyolojisi ve adli botanik uygulamaları açısından önem taşımaktadır. Ancak iğne yapraklı türlerden DNA izolasyonu, dokuların yüksek miktarda polisakkarit, polifenol, terpenoid ve reçine bileşikler içermesi nedeniyle güçleşmekte; bu bileşikler DNA izolasyonunu ve sonraki moleküler analizleri olumsuz etkileyebilmektedir. Bu çalışmada, Türkiye’de yaygın olarak bulunan *Pinus brutia* iğne yapraklarında örnek durumu ve homojenizasyon yönteminin elde edilen DNA konsantrasyonu üzerindeki etkilerinin değerlendirilmesi amaçlanmıştır. Bu kapsamda Çanakkale ilinde bulunan 24 farklı *P. brutia* ağacından iğne yaprak örnekleri toplanmıştır. On iki ağaçtan alınan örnekler taze olarak işlenirken, diğer 12 ağaçtan alınan örnekler laboratuvar koşullarında 20 gün süreyle kurutulmuştur. Her bir örnek durumu içerisinde örnekler, havanda öğütme sonrasında TissueLyser ile homojenizasyon ve blender ile homojenizasyon olmak üzere iki farklı yöntemle işlenmiştir. Genomik DNA, i-genomic Plant DNA Extraction Mini Kit kullanılarak izole edilmiş ve DNA konsantrasyonu Qubit™ 4 Florometre ile dsDNA High Sensitivity Assay kiti kullanılarak belirlenmiştir. Elde edilen veriler iki yönlü varyans analizi (ANOVA) ile değerlendirilmiştir. Taze örneklerde kurutulmuş örneklerle göre daha yüksek DNA konsantrasyonları elde edilirken, havanda öğütme sonrasında TissueLyser ile homojenizasyon uygulanan örneklerde blender ile homojenize edilen örneklerle göre daha yüksek DNA konsantrasyonları belirlenmiştir. İki yönlü ANOVA, örnek durumu ve homojenizasyon yönteminin DNA konsantrasyonu üzerinde anlamlı ana etkilerinin yanı sıra iki faktör arasında anlamlı bir etkileşim bulunduğunu göstermiştir. Bu bulgular hem örnek durumunun hem de homojenizasyon yönteminin *P. brutia* iğne yapraklarından elde edilen DNA konsantrasyonunu etkilediğini göstermekte ve moleküler bitki araştırmaları ile adli botanik uygulamalarında DNA ekstraksiyon prosedürlerinin örnek özelliklerine göre optimize edilmesinin önemini ortaya koymaktadır.

Anahtar Kelimeler: Bitki moleküler biyolojisi, adli botanik, DNA ekstraksiyonu, homojenizasyon, *Pinus brutia*

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1. Introduction

Obtaining sufficient DNA from plant tissues is fundamental for molecular taxonomy, phylogenetics, population genetics, biodiversity conservation, and forensic botany. DNA extraction constitutes the first and one of the most

critical steps for downstream molecular analyses, including DNA barcoding, microsatellite (SSR) analysis, single nucleotide polymorphism (SNP) genotyping, and sequencing-based approaches. Recent advances in molecular biology have substantially increased the

scientific value of plant-derived biological materials by enabling species identification even from fragmented or degraded samples (Lee et al., 2019; Oliveira et al., 2023).

Forensic science aims to reconstruct criminal events by establishing objective associations among victims, suspects, crime scenes, and physical evidence using validated scientific methods. Although biological evidence of human origin, such as blood, saliva, semen, and hair, remains important in forensic investigations, botanical evidence has gained increasing relevance owing to advances in molecular biology and DNA-based identification techniques (Coyle, 2004; Alotaibi et al., 2020; Oliveira et al., 2023).

Forensic botany integrates plant taxonomy, molecular genetics, ecology, palynology, dendrochronology, phytochemistry, and environmental sciences to support criminal investigations. Plant-derived evidence including leaves, needles, seeds, bark, roots, wood fragments, pollen, and spores can provide valuable information regarding the geographical origin of evidence, environmental conditions, postmortem relocation of human remains, and associations between suspects and crime scenes (Coyle, 2004; Houck & Siegel, 2022; Oliveira et al., 2023). Because plant materials are abundant, persistent, and readily transferred to clothing, footwear, vehicles, or other objects, they can serve as valuable trace evidence (Coyle, 2004).

Advances in molecular biology have considerably expanded the forensic value of plant evidence. DNA-based analyses can provide greater discriminatory power than conventional morphological examinations, particularly when botanical materials are fragmented, degraded, or lack diagnostic anatomical features. Consequently, molecular identification approaches, including DNA barcoding and other molecular markers, have become important tools for species identification and forensic interpretation (Alotaibi et al., 2020; Lee et al., 2019; Oliveira et al., 2023).

Successful molecular identification depends on obtaining sufficient DNA suitable for downstream analyses. However, DNA extraction from plants can be challenging because rigid cell walls and abundant polysaccharides, polyphenols, tannins, terpenoids, pigments, and other secondary metabolites may co-purify with DNA and interfere with downstream applications such as polymerase chain reaction (PCR), sequencing, and genotyping analyses (Domínguez-Rodríguez et al., 2020; Latorre et al., 2020; Särkinen et al., 2012; Wang et al., 2020). These challenges may be particularly relevant in conifer species because of their resinous and phenolic constituents and the structural characteristics of needle tissues (Kistler et al., 2020; Mofikoya et al., 2023). Therefore, sample homogenization and extraction strategy are important considerations in DNA isolation from plant tissues.

Various approaches have been developed to improve DNA isolation from plant tissues, including liquid nitrogen-assisted mortar grinding, bead-based homogenization systems, mechanical homogenizers, commercial silica membrane-based extraction kits, and modified cetyltrimethylammonium bromide (CTAB) protocols. Although commercial extraction kits can simplify laboratory workflows, their performance may vary according to sample matrix, tissue characteristics, and pretreatment procedures. Previous comparisons of DNA

isolation protocols in plant and plant-derived matrices have similarly demonstrated that extraction performance can vary depending on sample type, processing conditions, and the protocol applied (Ateş Sönmezoğlu & Terzi, 2019; Terzi Aksoy & Ateş Sönmezoğlu, 2022; Terzi Aksoy et al., 2024). Therefore, optimization of sample preparation and homogenization procedures remains important, particularly for matrices that present challenges for DNA isolation.

Among Mediterranean conifers, *Pinus brutia* (Turkish red pine) is an ecologically and economically important forest tree species. Türkiye harbors a substantial natural distribution of *P. brutia*, making this species relevant to forestry, biodiversity conservation, and potentially forensic investigations (Boydak, 2004; Kavgacı et al., 2021). Plant materials such as needles, bark, wood fragments, pollen, and cones may constitute botanical trace evidence in investigations involving forest environments and other outdoor crime scenes (Coyle, 2004; Oliveira et al., 2023).

Sample condition may also influence the amount of DNA obtained from plant material. Processes associated with drying and storage can affect plant tissues and may reduce the quantity or suitability of DNA available for molecular analysis (Oliveira et al., 2023; Maughan et al., 2023; Latorre et al., 2020). Accordingly, comparison of fresh and dried plant material can provide useful information for selecting appropriate sample preparation procedures.

The present study aimed to evaluate the effects of sample condition (fresh vs. dried) and homogenization method on DNA concentration obtained from *Pinus brutia* needles using a commercial silica membrane-based extraction kit. The study compared two homogenization approaches: mortar grinding followed by TissueLyser homogenization and blender homogenization.

The study was designed to determine how these factors influence DNA concentration and to provide data that may contribute to the optimization of DNA extraction procedures for *P. brutia* and related molecular plant and forensic botanical applications.

2. Materials and Method

2.1. Plant Material

Fresh needle samples of *Pinus brutia* were collected from 24 individual trees located in a forested area in Çanakkale, Türkiye (39.94° N, 26.93° E). To minimize cross-contamination, disposable gloves were changed before sampling each tree. Samples were individually placed into paper envelopes and transported to the laboratory. Samples collected from 12 trees were processed immediately as fresh samples, whereas samples collected from the remaining 12 trees were air-dried under laboratory conditions for 20 days prior to DNA extraction. Thus, the fresh and dried groups consisted of samples obtained from different individual trees.

2.2. Study Design

The study employed a 2 × 2 factorial design to evaluate the effects of sample condition and homogenization method on DNA concentration in *Pinus brutia* needles. A total of 24 samples obtained from 24 individual trees were analyzed, comprising 12 fresh and 12 dried needle samples. Each individual tree represented an independent biological replicate and experimental unit. Within each sample

condition, samples were equally assigned to two homogenization methods: mortar grinding followed by TissueLyser homogenization ($n = 6$) and blender homogenization ($n = 6$). Thus, the study comprised four experimental groups, with six independent biological replicates per sample condition $\times$ homogenization method combination.

2.3. Sample Homogenization

Fresh and dried *Pinus brutia* needles were homogenized using two different methods prior to DNA extraction.

For the mortar + TissueLyser method, approximately 50 mg of needle tissue from each sample was manually ground using a sterile porcelain mortar and pestle. For both fresh and dried samples, the ground tissue was subsequently subjected to additional homogenization using a TissueLyser LT (QIAGEN, Hilden, Germany) with stainless-steel beads following the manufacturer's protocol (QIAGEN, 2021).

For the blender method, approximately 50 mg of fresh or dried needle tissue was mechanically homogenized using a Philips Avent SCF870 blender (Philips, Amsterdam, Netherlands). The homogenized material was immediately used for DNA extraction.

2.4. DNA Extraction

Genomic DNA was extracted from all homogenized samples using the i-genomic Plant DNA Extraction Mini Kit (iNtRON Biotechnology Inc., Seongnam, Republic of Korea) following the manufacturer's protocol (iNtRON Biotechnology Inc., 2021). Fresh and dried samples were processed using the same extraction procedure to ensure consistency among experimental groups. For the final elution step, the spin column was transferred to a new 1.5-mL microcentrifuge tube, and 100 μ L of PE buffer was applied directly to the membrane. After incubation for 2 min, DNA was eluted by centrifugation at 13,000 rpm for 2 min. The eluted DNA was stored at -20°C until DNA quantification.

2.5. DNA Quantification

DNA concentration was determined using a Qubit™ 4 Fluorometer together with the Qubit™ dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations (Thermo Fisher Scientific, 2020). DNA concentrations (ng/ μ L) obtained from each sample were recorded and used for subsequent statistical analyses.

2.6. Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics (Version 29.0; IBM Corp., Armonk, NY, USA). Data normality was evaluated using the Shapiro–Wilk test. The effects of sample condition (fresh vs. dried), homogenization method (mortar grinding followed by

TissueLyser vs. blender), and their interaction on DNA concentration were evaluated using a two-way analysis of variance (ANOVA). Data are presented as mean $\pm$ standard deviation (SD), and statistical significance was set at $p < 0.05$.

3. Results

A total of 24 *Pinus brutia* needle samples were analyzed, comprising 12 fresh and 12 dried samples. Within each sample condition, six samples were homogenized using mortar grinding followed by TissueLyser homogenization, whereas the remaining six samples were homogenized using blender homogenization.

The highest DNA concentration was obtained from fresh needle samples processed by the mortar grinding–TissueLyser protocol (14.89 ± 8.04 ng/ μ L), whereas fresh samples homogenized using the blender yielded substantially lower DNA concentrations (1.42 ± 1.57 ng/ μ L). Likewise, dried needles processed using the mortar grinding–TissueLyser method produced higher DNA concentrations (4.31 ± 3.31 ng/ μ L) than those processed using the blender (0.40 ± 0.68 ng/ μ L) (Table 1).

The distribution of individual DNA concentrations across the four experimental groups is presented in Figure 1.

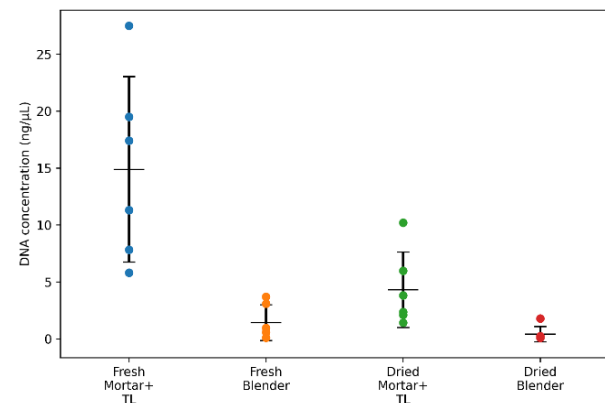


Figure 1. DNA concentrations in fresh and dried *Pinus brutia* needles following mortar grinding combined with TissueLyser homogenization or blender homogenization

Statistical significance was assessed using two-way ANOVA. Two-way ANOVA demonstrated significant main effects of both sample condition ($F(1,20) = 10.046$, $p = 0.0048$) and homogenization method ($F(1,20) = 22.555$, $p < 0.001$). A significant interaction between sample condition and homogenization method was also observed ($F(1,20) = 6.834$, $p = 0.0166$), indicating that the effect of homogenization method on DNA concentration differed according to sample condition (Table 2).

Overall, fresh needle samples showed higher DNA concentrations than dried samples, while mortar grinding followed by TissueLyser homogenization resulted in higher

Table 1. DNA concentrations obtained from fresh and dried *Pinus brutia* needles following different homogenization methods ($N = 24$, $n = 6$ per group)

Sample condition	Homogenization method	n	DNA concentration (ng/ μ L), Mean $\pm$ SD
Fresh	Mortar grinding + Tissue Lyser	6	14.89 ± 8.04
Fresh	Blender	6	1.42 ± 1.57
Dried	Mortar grinding + Tissue Lyser	6	4.31 ± 3.31
Dried	Blender	6	0.40 ± 0.68

Table 2. Two-way ANOVA evaluating the effects of sample condition and homogenization method on DNA concentration

Source	Df	F	p	Partial η^2
Sample condition (Fresh vs. Dried)	1	10.046	0.0048	0.334
Homogenization method (Mortar + TissueLyser vs. Blender)	1	22.555	<0.001	0.530
Condition $\times$ Method	1	6.834	0.0166	0.255
Residual	20	—	—	—

DNA concentrations than blender homogenization. The significant interaction indicates that the magnitude of the homogenization effect differed between fresh and dried samples.

4. Discussions

Obtaining sufficient DNA is a prerequisite for molecular analyses in plant biology and provides a foundation for downstream applications such as DNA barcoding, microsatellite (SSR) genotyping, SNP analysis, and DNA sequencing. In forensic botany, where biological evidence may be limited or exposed to environmental conditions before collection, optimization of DNA extraction procedures is particularly important (Oliveira et al., 2023; Paranaíba et al., 2020). The present study demonstrated significant effects of both sample condition and homogenization method on DNA concentration obtained from *Pinus brutia* needles. Fresh needles showed higher mean DNA concentrations than dried needles, while mortar grinding followed by TissueLyser homogenization resulted in higher mean DNA concentrations than blender homogenization. Furthermore, the significant interaction between sample condition and homogenization method indicates that the effect of homogenization method on DNA concentration differed according to sample condition. These findings emphasize the importance of considering both tissue characteristics and sample condition when optimizing DNA extraction procedures rather than applying a single universal strategy (Kistler et al., 2020; Mofikoya et al., 2023; Paranaíba et al., 2020).

Fresh needles showed higher DNA concentrations than dried needles. Drying and storage may alter plant tissues and can affect the quantity and suitability of DNA obtained for molecular analysis. Dehydration, oxidative processes, DNA fragmentation, and changes in the relative abundance of phenolic compounds, polysaccharides, resinous substances, and other secondary metabolites may contribute to difficulties in DNA isolation from dried plant material (Latorre et al., 2020; Särkinen et al., 2012; Wang et al., 2020).

Similar challenges have been reported for woody plant materials, for which optimization of DNA extraction procedures may be required to address species- and tissue-specific biochemical characteristics (Mitchell et al., 2023). Nevertheless, measurable DNA concentrations were obtained from all dried needle samples examined in the present study. These findings indicate that DNA can be obtained from dried *P. brutia* needles; however, the suitability of the extracted DNA for downstream molecular analyses requires further evaluation. The applicability of the approach to environmentally exposed samples should also be investigated in future studies.

Among the investigated factors, homogenization method showed the largest effect size for DNA concentration, as indicated by the partial η^2 value obtained from the two-way

ANOVA. Efficient disruption of plant tissues is important because the cellulose-rich cell wall, lignified tissues, and secondary metabolites can limit access to intracellular DNA during extraction (Domínguez-Rodríguez et al., 2020; Särkinen et al., 2012).

Previous comparative studies have similarly shown that DNA extraction performance can vary according to both sample matrix and the isolation protocol employed. Differences among DNA isolation procedures have been reported in wheat and processed plant-derived food matrices, highlighting the importance of matrix-specific optimization rather than reliance on a single extraction procedure for different sample types (Ateş Sönmezoğlu & Terzi, 2019; Terzi Aksoy & Ateş Sönmezoğlu, 2022; Terzi Aksoy et al., 2024).

These considerations may be particularly relevant to conifer tissues. Unlike soft parenchymatous tissues, *Pinus brutia* needles possess structural characteristics that can increase resistance to mechanical disruption, while resinous and phenolic constituents may further complicate DNA isolation (Boydak, 2004; Mofikoya et al., 2023).

The higher DNA concentrations observed following mortar grinding combined with TissueLyser homogenization may therefore reflect more effective mechanical disruption of needle tissues compared with blender homogenization. The significant interaction between sample condition and homogenization method further indicates that the magnitude of the homogenization effect differed between fresh and dried samples. Although mean DNA concentrations were lower in dried samples with both homogenization procedures, the difference between the two homogenization methods was greater in fresh samples. These findings suggest that both sample condition and tissue disruption procedure should be considered when selecting a DNA extraction workflow for *P. brutia* needles.

The present findings are consistent with those reported by Ünsal Sapan et al. (2025) for *Pyracantha coccinea*, where mortar grinding combined with TissueLyser homogenization resulted in higher DNA concentrations than blender homogenization. Although similar effects of tissue disruption were observed in the two studies, differences in the magnitude of the homogenization effect may be related to the anatomical characteristics of the plant materials. Firethorn fruits consist predominantly of soft parenchymatous tissue, whereas pine needles have a more rigid structure that may increase resistance to mechanical homogenization. These observations further emphasize the importance of considering plant species and tissue characteristics when selecting homogenization procedures for DNA extraction. In addition, measurable DNA concentrations were obtained without the use of liquid nitrogen in both studies, suggesting that mortar grinding combined with TissueLyser homogenization may provide a practical alternative for laboratories without access to

cryogenic homogenization facilities (Särkinen et al., 2012; Ünsal Sapan et al., 2025).

Beyond forensic applications, optimization of DNA extraction procedures for *P. brutia* may also be relevant to molecular taxonomy, forest genetics, biodiversity conservation, and ecological research. *Pinus brutia* is an ecologically and economically important conifer species in Türkiye and the eastern Mediterranean region (Boydak, 2004).

DNA extraction procedures applicable to fresh and dried needles may support subsequent applications including DNA barcoding, population genetic analyses, SSR and SNP genotyping, phylogeographic investigations, and next-generation sequencing. However, because the present study evaluated DNA concentration rather than downstream molecular performance, the suitability of the extracted DNA for these applications should be confirmed through further analyses (Corradini et al., 2024; Lee et al., 2019; Oliveira et al., 2023).

Several limitations should be acknowledged. Only a single silica membrane-based extraction kit was evaluated, and DNA assessment was limited to concentration measured using the Qubit fluorometric assay. DNA purity, integrity, amplification performance, and sequencing suitability were not evaluated. In addition, the dried samples were prepared under controlled laboratory conditions and therefore do not represent the full range of environmental exposures that may affect botanical evidence in forensic contexts. Future studies should compare alternative extraction chemistries, assess DNA purity and integrity, evaluate amplification success using PCR or quantitative PCR, and investigate sequencing performance. Additional conifer species and samples exposed to different environmental conditions should also be examined to determine the broader

applicability of the homogenization approach (Corradini et al., 2024; Mitchell et al., 2023; Sugita et al., 2020).

Overall, the present study demonstrates that DNA concentration obtained from *Pinus brutia* needles is influenced by both sample condition and homogenization method. Mortar grinding followed by TissueLyser homogenization resulted in the highest mean DNA concentration among the four experimental groups and represents a practical approach for DNA extraction from fresh and dried needle tissues without the need for liquid nitrogen. Together with previous findings in *Pyracantha coccinea* (Ünsal Sapan et al., 2025), the present results further emphasize the importance of optimizing DNA extraction procedures according to plant species, tissue characteristics, and sample condition rather than relying on a generalized extraction strategy. The findings provide a basis for further evaluation of this approach in molecular plant biology, forest genetics, biodiversity conservation, and forensic botany. Future studies evaluating DNA purity, integrity, amplification success, and sequencing performance in environmentally exposed samples and additional conifer species are required to establish its broader applicability.

Conflict of Interest

No Conflict of Interest

Authors' Contribution

NGT performed sample collection, laboratory experiments, and data collection, while TUS conceived and designed the study, supervised the research, performed the data analysis, interpreted the results, and wrote and revised the manuscript. All authors read and approved the final manuscript.

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