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THE INFLUENCE OF STORAGE CONDITIONS AND TOTAL DNA EXTRACTION PROTOCOL ON THE RESULTS OF MOLECULAR ANALYSIS OF THE EUROPEAN SPRUCE BARK BEETLE (*Ips typographus* L.)

VPLIV POSTOPKOV SHRANJEVANJA IN EKSTRAKCIJE CELOKUPNE DNA NA REZULTATE MOLEKULARNE ANALIZE OSMEROZOBEGA SMREKOVEGA LUBADARJA (*Ips typographus* L.)

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ABSTRACT

One of the key steps of the molecular identification of bark beetles is obtaining a sufficient quantity of high-quality DNA extract. In this study, we investigated the influence of different storage procedures for *Ips typographus* (L.) specimens and various DNA extraction protocols on the quantity and quality of DNA intended for use in molecular diagnostics. Adult beetles were frozen at -20 °C, either dry or in ethanol. We tested four different protocols for DNA extraction. We compared the quantity of extracted DNA and assessed its quality with PCR and Sanger sequencing. Different storage protocols had no significant effect on the quantity of DNA extracted. However, freezing specimens in ethanol provided higher-quality DNA for molecular applications. Only two of the extraction protocols produced sequenceable amplicons, and the difference in the amount of extracted DNA between them was not significant. We propose the optimal combination of storing specimens in ethanol at -20°C and using the Nucleospin Insect DNA extraction kit from Macherey Nagel, enabling a time-efficient identification process.

Key words: early detection, specimen storage, total DNA extraction, PCR, polymerase chain reaction, Sanger sequencing, molecular diagnostics

IZVLEČEK

Za uspeh molekularne identifikacije podlubnikov je ključna zadostna količina kakovostnega ekstrakta DNA. Pri raziskavi smo se osredotočili na vpliv različnih postopkov shranjevanja osebkov *Ips typographus* (L.) in različnih protokolov ekstrakcije DNA na količino in kakovost DNA, namenjene za uporabo v molekularni diagnostiki. Odrasle hrošče smo zamrznili pri -20 °C na dva načina, v etanolu in na suho. Ekstrakcije smo opravili po štirih različnih postopkih. Primerjali smo količino pridobljene DNA. Kakovost ekstrahirane DNA smo ocenili s polimerazno verižno reakcijo in sekvenciranjem po Sangerju. Način shranjevanja ni statistično značilno vplival na količino pridobljene DNA. DNA višje kakovosti smo pridobili iz hroščev, ki so bili zamrznjeni v etanolu. Le dva postopka ekstrakcije DNA sta dala DNA, uporabno za sekvenciranje. V količini pridobljene DNA se pri sekvenciranju uspešna postopka nista pomembno razlikovala. Določili smo optimalno kombinacijo postopkov shranjevanja v etanolu pri -20 °C in ekstrakcije celokupne DNA z ekstrakcijskim kitom Nucleospin Insect DNA proizvajalca Macherey Nagel, ki omogoča časovno učinkovito identifikacijo.

Ključne besede: zgodnje zaznavanje, shranjevanje osebkov, ekstrakcijski postopek celokupne DNA, PCR, polimerazna verižna reakcija, sekvenciranje po Sangerju, molekularna diagnostika

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1 INTRODUCTION
1 UVOD

Bark beetles (Coleoptera: Curculionidae: Scolytinae) play a crucial role in forest ecosystems. The vast majority of species breed in dead and dying woody plant tissues, thus contributing to the cycling of organic matter (Machingambi et al., 2014). However, some bark beetle species (e.g., *I. typographus* L., *Pityogenes chalcographus* B., *I. duplicatus* S.) can also cause significant ecological and economic damage in the form of tree mortality following exceptional climatic events (e.g., windthrow, snow damage, ice storms, and drought)

(Avtzis et al., 2012; de Groot et al., 2018; Gandhi and Hofstetter, 2021). Worldwide, there are more than 6000 species of bark beetles (Linnakoski et al., 2012; Faccoli, 2015), of which around 90 are found in Slovenia, with new reports continuously emerging (Titovšek, 1988; Jurc, 2003; Kavčič, 2018; Hauptman et al., 2019).

The European spruce bark beetle (*I. typographus*) can cause considerable damage to Norway spruce (Gandhi and Hofstetter, 2021; Hlásny et al., 2021), which is one of the most economically important tree species in Europe (San-Miguel-Ayanz et al., 2022). During the past century, Norway spruce (*Picea abies*, (L.) H. Karst.) has been extensively planted outside its natural distribution range, where growing conditions for this species are suboptimal. With a warming climate and unfavourable weather events inducing higher stress and providing better living conditions for bark beetle development, there has been an increase in the frequency and extent of outbreaks (Netherer and Hammerbacher, 2022). Identifying species morphologically or with molecular methods is often the first step in developing plans to manage such outbreaks and is an important part of the early detection of native as well as invasive alien species.

The early detection of new and emerging pests is essential to prevent and limit damage, while correct identification is crucial for the implementation of appropriate control measures to further reduce the risk posed by pests (Morales-Rodríguez et al., 2019; Poland and Rassati, 2019). Since measures, and therefore costs, are proportional to the threat posed by the identified species to the local ecosystem, incorrect identification can result in unnecessary measures with high economic and ecological costs (Lima et al., 2022; Chen et al., 2023). Historically, morphological identification was mainly used (Madden et al., 2019). With the rapid spread and introduction of species globally, taxonomists are facing a growing challenge (Poland and Rassati, 2019). In recent decades, molecular methods have developed rapidly to aid in the identification of pests and other species (EPPO, 2021; Madden et al., 2019). Additionally, morphological methods are often limited to undamaged adult specimens, whereas molecular methods enable identification even from samples with missing morphological features and juvenile stages, sometimes even from materials containing only traces of the target DNA (e.g., frass, swabs from galleries) (Boykin et al., 2012; Taylor and Harris, 2012; Jörger and Schrödl, 2013; Albo et al., 2019; Morales-Rodríguez et al., 2019; Poland and Rassati, 2019), often referred to as environmental DNA, or eDNA (Ficetola et al., 2008; Taberlet et al., 2012; Gorički et al., 2017).

Storage of samples prior to molecular analysis is an important part of the analytical process and can influence the final result of the analysis (Ballare et al., 2019; Whitman et al., 2019; Martoni et al., 2021; Moškrič et al., 2023). The choice of DNA extraction protocol is also important, as it can influence the quantity and quality of the extracted DNA (Wang et al., 2019). Additionally, the presence of (ideally well-preserved) DNA in the sample is critical to the success of the extraction (Nagy, 2010). In addition to oxidation, the presence of water and high temperatures are the main causes of DNA degradation, as they promote enzymatic activity, with nuclease-type enzymes being the most problematic for DNA preservation (Gemeinholzer et al., 2010; Nagy, 2010). Such conditions result in oxidative damage to the DNA molecule, molecular crosslinking, and fragmentation (Deagle et al., 2006), making the damaged DNA less useful for PCR and other molecular methods. For animal tissues, storage of DNA in a high concentration of ethanol is usually recommended (Gemeinholzer et al., 2010).

In the present study, we compared two different ways of storing *I. typographus* specimens at -20°C: (i) dry storage and (ii) storage in 96% ethanol. Both storage protocols are relatively undemanding in terms of laboratory equipment, as they only require access to a standard freezer and require no elaborate preparation apart from drying the specimen on filter paper in order to remove the ethanol prior to DNA extraction. In addition, we tested four different total DNA extraction protocols based on commercial extraction kits from two different manufacturers. The selected kits are all commercially available, time-efficient, and easy to use in a laboratory setting. We measured variables such as the concentration and calculated the final amount of total DNA in the extracts. We used endpoint PCR and subsequent Sanger sequencing of the amplicons to assess the quality of the extracted DNA and its usefulness in molecular identification. For the purposes of this study, DNA was deemed useful for molecular identification if it could be used to produce a PCR amplicon that could in turn be sequenced, and the resulting chromatograms were of sufficient quality to enable reliable identification of the target via pairwise alignment to a reference sequence. Our results provide guidelines for the storage of specimens and the choice of total DNA extraction protocol for small arthropod samples intended for molecular analysis.

2 METHODS
2 METODE

In May and June 2021, adult bark beetles were collected from Norway spruce logs kept in rearing tents.

Table 1: List of protocols and commercial kits used for total DNA extraction

Preglednica 1: Seznam postopkov in komercialnih kitov, ki so bili uporabljeni za ekstrakcijo celokupne DNA

Protocol name	Kit and protocol used
P1	Nucleospin DNA Insect Kit (Macherey Nagel, Düren, Germany)
P2	DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands)
P3	Qiagen QIAamp DNA Micro Kit (Qiagen, Venlo, Netherlands) without carrier RNA in the binding buffer
P4	Qiagen QIAamp DNA Micro Kit (Qiagen, Venlo, Netherlands) with carrier RNA in the binding buffer

Bark beetles were morphologically identified to the species level at the Laboratory of Forest Protection. Forty-eight specimens, identified as *Ips typographus*, were frozen, either dry or stored in 96% ethanol.

After one month of storage, four different protocols were used to extract total DNA from individual bark beetle specimens according to the manufacturers' instructions (Table 1). For each extraction protocol, total DNA was extracted from three individual adult specimens per storage protocol, i.e., 12 adult specimens per storage protocol.

The concentration of resulting total DNA extracts was quantified with a Biophotometer Plus (Eppendorf, Germany) and µCuvette (Eppendorf, Germany). DNA quantity was then calculated from the concentration of DNA and volume of the extracts.

We tested two different sets of primers targeting the arthropod COI gene with an end-point PCR. The LCO1490/HCO2198 primer pair was developed by Folmer et al. (1994) and the Ips_F_cons/Ips_R_cons pair was developed by Becker et al. (2021) (Table 2).

The PCR master mix was prepared using Amplitaq Gold 360 MM (ThermoFisher Scientific, Massachusetts, USA): 25 µl of master mix, 1 µl of GC Enhancer, 1 µl of each primer, and 4 µl DNA (standardized to a concentration of 10 ng/µl) per 50 µl reaction. PCR products were separated on a 1% agarose gel, and in the case of the successful amplification of a product of expected size, purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA) and then sequenced at a sequencing facility (Eurofins, Köln, Germany) in both forward and reverse directions using the same primers as for the PCR. Sequences were visualized and manually edited using Geneious Prime® v.2021.2.2. (Biomatters Ltd., Auckland, New Zealand). The obtained consensus sequences were compared to reference sequence MK314170 using MAFFT Alignment implemented in Geneious Prime and deposited at GenBank.

The amount of DNA extracted was normalized to the amount of input material. The normalized amount of DNA extracted from beetles stored in two different ways and using three different DNA extraction protocols was then compared using the statistical software R version 4.2.2 (R Core Team, 2022). In Protocol 4, the RNA carrier is used in the binding buffer during extraction, resulting in residual RNA presence in the final DNA extract. UV-VIS photometry was used for the quantification of DNA extracts. As the method cannot discriminate between RNA and DNA in the sample, the actual concentration of the extracts produced using Protocol 4 is lower than the one measured. For this reason, data produced with Protocol 4 were not included in the statistical analysis of the amount of DNA extracted using the different protocols. Due to the non-normality of the acquired data, the Wilcoxon rank-sum test was used to check for differences between the two storage protocols. For differences between the extraction protocols, the non-parametric Kruskal-Wallis test was used, followed by the Dunn post-hoc test. The statistical analysis and the creation of graphs were performed using the packages "FSA" (Ogle et al., 2022), "ggpubr" (Kassambara, 2022), and "ggplot2" (Wickham, 2016).

3 RESULTS
3 REZULTATI

The average mass of *I. typographus* specimens used for total DNA extraction was 10 mg per extraction.

The calculated average amount with the standard deviation of total DNA extracted per gram of input

Table 2: Primers and conditions used in the study (Folmer et al., 1994; EPPO, 2021; Becker et al., 2021)

Preglednica 2: Začetni oligonukleotidi in pogoji, uporabljeni v raziskavi (Folmer et al., 1994; EPPO, 2021; Becker et al., 2021)

Primers	Primer sequence (5'-3')	Annealing temperature	Expected amplicon length
LCO1490 HCO2198	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	45°C (5 cycles) + 51°C (25 cycles)	709 bp
Ips_F_cons Ips_R_cons	CAAATATTGCCCATGAAGGAAC GCATCTGGATAATCTGAGTAACGTCG	55°C (35 cycles)	936 bp

Table 3: Average amount and the standard deviation of total DNA (µg) extracted from one gram of specimen with the different extraction protocols and storage conditions. P1: Nucleospin DNA Insect Kit (Macherey Nagel, Düren, Germany); P2: DNeasy Blood and Tissue Kit (Qiagen, Venlo, Netherlands); P3: Qiagen QIAamp DNA Micro Kit (Qiagen, Venlo, Netherlands) without carrier RNA in the binding buffer; P4: Qiagen QIAamp DNA Micro Kit (Qiagen, Venlo, Netherlands) with carrier RNA in the binding buffer.

Preglednica 3: Povprečna količina in standardni odklon celokupne DNA (µg), pridobljene iz enega grama osebka glede na uporabljen postopek in način shranjevanja. P1: Nucleospin DNA Insect Kit (Macherey Nagel, Düren, Nemčija); P2: DNeasy Blood and Tissue Kit (Qiagen, Venlo, Nizozemska); P3: Qiagen QIAamp DNA Micro Kit (Qiagen, Venlo, Nizozemska) brez nosilne RNA v pufru za vezavo; P4: Qiagen QIAamp DNA Micro Kit (Qiagen, Venlo, Nizozemska) z nosilno RNA v pufru za vezavo.

Storage protocol	Total DNA extraction protocol			
	P1	P2	P3	P4
Frozen	191 (± 39)	414 (± 112)	175 (± 50)	405 (± 89)
Frozen in EtOH	314 (± 80)	618 (± 518)	148 (± 28)	376 (± 4)

specimen was 191 ± 39 µg from frozen and 314 ± 80 µg from frozen in ethanol with the P1 extraction protocol, 414 ± 112 µg from frozen and 618 ± 518 µg from frozen in ethanol with the P2 extraction protocol, 175 ± 50 µg from frozen and 148 ± 28 µg from frozen in ethanol with the P3 extraction protocol, and 405 ± 89 µg from frozen and 376 ± 4 µg from frozen in ethanol with the P4 extraction protocol (Table 3).

It was demonstrated that there was a significant difference between the amount of DNA extracted per gram of input specimen among the three assessed protocols, i.e., P1, P2, and P3 (Table 4). After conducting the Dunn post-hoc test, we found that only the use of P2 and P3 produced significantly different amounts of total DNA (Fig. 1, Table 4). The minimum/maximum amount of DNA produced from one gram of input specimen by P2 was 285 µg/480 µg for the frozen storage protocol and 268 µg/1213 µg for the samples frozen in ethanol. The P3 extraction protocol produced a minimum of 127 µg and a maximum of 226 µg of DNA per one gram of the frozen samples and a minimum of 115 µg and a maximum of 167 µg of DNA per one gram of the samples frozen in ethanol. The P1 protocol was not indicated as producing significantly different amounts compared to both the P2 and P3 protocols (Fig. 1, Table 4).

Using the Wilcoxon rank-sum test with continuity correction, no significant difference in the amount of DNA extracted between the storage protocols was found ($df = 1$, $W = 35$, $p = 0.667$).

Using LCO1490/HCO2198 primers on the total DNA extracts from *I. typographus* specimens did not produce any amplicons with most of the samples used. Only two out of 24 DNA extracts tested provided useful sequences, with both obtained consensus sequences belonging to parasitoid nematodes from the order Rhabditidae (results not shown).

PCR using primers Ips_F_cons/Ips_R_cons were more successful, as most of the protocols we tested produced amplicons of expected size around 936 bp (Fig. 2). However, only amplicons produced using protocols P1 and P2 were of sufficient quantity and quality to enable successful sequencing (Table 5).

4 DISCUSSION AND CONCLUSIONS
4 RAZPRAVA IN ZAKLJUČKI

Our study confirmed that the choice of specimen storage method and total DNA extraction protocol can significantly affect the outcome of downstream molecular processes in the molecular identification of *Ips typographus*. When analyzing the amount of DNA extracted per gram of input material with each extraction protocol, the only significant difference was found between the P2 and P3 protocols (Fig. 1, Table 4), while the amount of DNA extracted per gram of input material was not significantly affected by the different storage methods (Fig. 1). PCR using universal arthropod

Table 4: Results of the Kruskal-Wallis and the Dunn post-hoc tests for comparing the amount of DNA extracted per gram of specimen with the extraction protocols P1, P2, and P3

Preglednica 4: Rezultati Kruskal Wallis-ovega in Dunn-ovega post-hoc testa za primerjavo količine ekstrahirane DNA iz enega grama osebka s postopki P1, P2 in P3

Kruskal Wallis test			Dunn post-hoc test	
Chi square (χ^2)	Degrees of freedom	*P* value	Comparison	P value
10.89	2	0.004	P1-P2	0.251
			P1-P3	0.351
			P2-P3	0.003

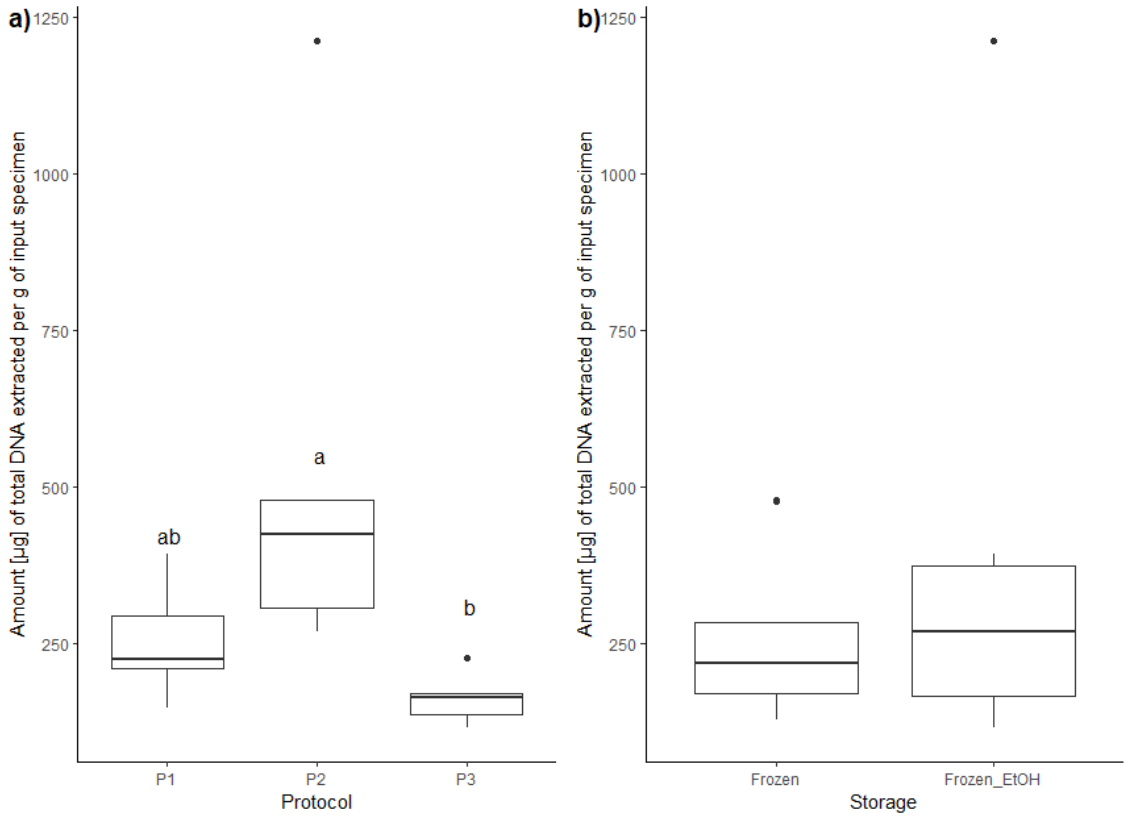

Fig. 1: Amount of total DNA (µg) extracted per gram of specimen by a) extraction protocol and b) storage method used

Slika 1: Količina ekstrahirane celokupne DNA (µg) iz enega grama osebka glede na a) ekstrakcijski postopek in b) način shranjevanja

primers LCO1490/2198 was not successful in amplifying *I. typographus* DNA. The use of Ips_F_cons/Ips_R_cons primers enabled successful amplification across all storage methods and extraction protocols (Fig. 2), although gel bands produced by extracts from extraction protocols P3 and P4 were only slightly visible, and only extraction protocols P1 and P2 enabled successful sequencing of the products (Table 5).

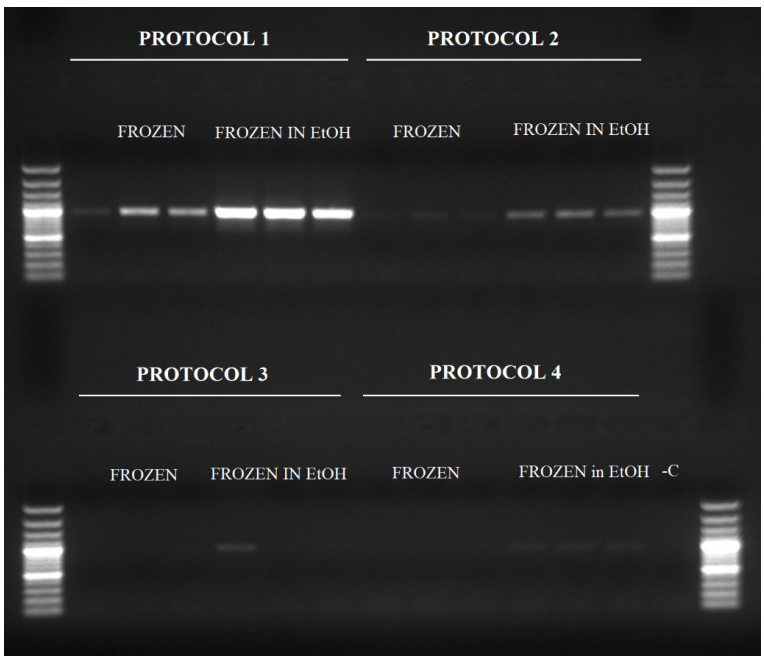

Fig. 2: Amplicons from the PCR using Ips_F_cons/Ips_R_cons after separation (100 bp ladders used)

Slika 2: Pomnožki, pridobljeni v polimerazni verižni reakciji z začetnima oligonukleotidoma Ips_F_cons/Ips_R_cons po ločevanju na gelu (uporabljena je bila lestvica 100 bp)

Table 5: Results of Sanger sequencing and subsequent chromatogram analysis of *Ips typographus* specimens using Ips_F_cons/Ips_R_cons primers (F – frozen, F+EtOH – frozen in ethanol), including the GenBank Accession Numbers of the obtained sequences

Preglednica 5: Rezultati sekvenciranja osebkov *Ips typographus* po Sangerju z uporabo začetnih oligonukleotidov Ips_F_cons/Ips_R_cons in sledeče analize kromatogramov (F – zamrznjen, F+EtOH – zamrznjen v etanolu), vključno z GenBank akcesijskimi številkami pridobljenih sekvenc

Extraction protocol	Storage protocol	PCR product concentration [µg/ml]	Consensus sequence	Length (trimmed)	%HQ	% Identity to ref. sequence (MK315170)	GenBank Accession Number
P1	F	66.8	no	-	-	-	-
P1	F	68.6	yes	889	94.0	99.8	OR458594
P1	F	60.9	yes	889	97.6	99.9	OR458590
P1	F + EtOH	80.2	yes	885	98.4	99.8	OR458592
P1	F + EtOH	81.3	yes	878	96.2	99.9	OR458591
P1	F + EtOH	83.2	yes	851	91.2	99.9	OR458595
P2	F	59.7	no	-	-	-	-
P2	F	73.1	no	-	-	-	-
P2	F	64.1	no	-	-	-	-
P2	F + EtOH	64.2	no	769	81.0	99.7	OR458593
P2	F + EtOH	83.7	yes	889	90.2	99.7	OR458589
P2	F + EtOH	61.6	no	-	-	-	-
P3	F	64.3	no	-	-	-	-
P3	F + EtOH	60.7	no	-	-	-	-
P3	F + EtOH	59.8	no	-	-	-	-
P4	F + EtOH	74.3	no	-	-	-	-
P4	F + EtOH	60.4	no	-	-	-	-
P4	F + EtOH	65.6	no	-	-	-	-

When choosing a storage protocol for arthropod specimens, one of the factors that should be considered is the usability of the samples for downstream processing. For specimens intended for morphological identification, dry freezing may not be suitable for long-term storage, as over time they may become too dehydrated to remain useful for morphological examination (Whitman et al., 2019). However, for specimens intended for use in downstream molecular processes, one of the key considerations is the preservation of DNA quality. While storage in 96% ethanol may also be problematic for morphological applications (Quicke et al., 1999), it is generally considered suitable for use with specimens intended for downstream molecular applications, provided that the ethanol used is non-methylated (Whitman et al., 2019).

While our data indicates no difference in the amount of DNA extracted per gram of input material between the two different storage protocols, there is evidence of a difference in the rate of DNA degradation between the two protocols. In Fig. 2, there is a clear visual difference between the bands produced by extracts from specimens that were frozen dry and those frozen in ethanol. Since the extracts used were normalized to a concentration of 10 ng/µl, the difference in bands was not due to the starting amount of DNA but to its suitability for use in PCR. The difference in in DNA degradation between the two storage protocols may be due to the fact that ethanol denatures proteins, including those with DNase activity (Flournoy et al., 1996; Stein et al., 2013). While this could be disadvantageous as it also denatures histones and other structural proteins, which might consequently make the DNA structurally inaccessible for extraction, we resolved this by choosing extraction protocols that include the use of a proteinase. Another possible reason for the difference in degradation may be that ethanol also displaces water in tissues, thus preventing the water-mediated DNA degradation that would otherwise occur during dry storage, where water that is inherently present remains in the cells (Stein et al., 2013). Ice crystals, which can form when water is present in the cells during freezing, further exacerbate this degradation effect. The storage protocol without ethanol produced only two readable sequences in our results, compared to five produced by specimens frozen in ethanol. In all seven cases, the consensus sequences were sufficiently identical to the reference sequence to enable identification of the specimens. The amplicon produced by the Ips_F_cons and Ips_R_cons primers is relatively long,

at 936 bp. If the specimens were intended for downstream applications using primers that produced amplicons of shorter length, the effect of storage should be even less pronounced.

During our study, DNA belonging to endoparasites was also amplified in the tested samples. It is known that species of the *Ips* genus often host endoparasites (Hoffard and Coster, 1976), which are also included in the broad target spectrum of the general LCO1490/HCO2198 primers. However, host-specific primers are more useful for molecular identification in the presence of endoparasites. As previously proposed (Becker et al., 2021), the results demonstrate the limitations of using universal primer pairs such as LCO1490/HCO2198 with some arthropod samples. This approach might work for other Scolytinae taxa without the problems of non-target amplification, as demonstrated by Marinč et al. (2019). In their study, five kits for total DNA extraction on beetles of the genus *Ambrosiodmus* were compared, including DNeasy Blood and Tissue and Nucleospin DNA Insect, which were also used in our study. The authors found these two kits to be the most useful of the five kits tested in terms of DNA yield for the DNeasy Blood and Tissue Kit, and time- and cost- efficiency for the Nucleospin DNA Insect Kit. Both kits also performed well in our comparisons, although the amount of DNA extracted was not significantly different between the two kits. The DNA extracted with the Nucleospin DNA Insect Kit proved to be more useful for sequencing applications (Table 5). In conclusion, both kits performed well in the PCR on different Scolytinae taxa in both studies, although our results indicate the importance of verifying the kits for sequencing applications, as sequencing was not equally successful using extracts from different kits.

Protocols P3 and P4 both include overnight lysis at 56°C, which may also contribute to DNA degradation, explaining why the PCR using extracts from these two protocols was less successful, yielding only slightly visible bands (Fig. 2) and no useful sequencing results (Table 5). However, Rohland (2007) proposed, based on ancient DNA, that the increased DNA degradation and reduced proteinase K activity over time in longer lysis steps are compensated for by the greater DNA release over time.

In our study, the volumes of elution buffers were standardized across the protocols (100 µl). As the amount of starting material was quite low (average weight of 10 mg per *I. typographus* specimen), reducing the volume of elution buffer and multiple elution steps with the same buffer could be explored in further studies, as this may improve the amount of DNA eluted.

As the chosen kits were all based on silica column extraction with a similar chemical background, further research should be performed to include different types of extraction methods, such as solution-based CTAB (Saghai-Maroof et al., 1984; Doyle and Doyle, 1987) and phenol-chloroform (Chomczynski and Sacchi, 1987) extractions or resin-based Chelex extractions (Walsh et al., 1991). Additionally, manual or automatic extraction methods based on magnetic or paramagnetic beads (Alderton et al., 1992) should be tested. Additionally, including more samples for each treatment would make the results even more statistically robust.

The choice of protocols for specimen storage prior to extraction is important and should be tailored to the intended downstream applications. Based on our results, freezing specimens in ethanol produced similar amounts of DNA as dry freezing, although the former proved more suitable for sequencing applications. Similarly, the choice of commercially available extraction kits and protocols should be based on evidence demonstrating their suitability for the specific type of samples and downstream applications, as different protocols do not produce similar results on the same type of samples. Of the four DNA extraction protocols tested, Protocol 1 using the Nucleospin DNA Insect Kit (Macherey Nagel, Düren, Germany) proved to be the most useful for relatively small arthropod samples intended for PCR and sequencing applications. As observed, different extraction protocols produce DNA extracts of varying quality in terms of their usefulness for sequencing applications. Molecular methods are gaining popularity for the identification of existing and emerging forest pest species, as the time component is crucial, especially in the early detection of invasive species. Because such methods rely on obtaining a sufficient quantity and quality of the target DNA, this work represents a crucial step in the process of ensuring reliable results and thus improving the tools needed to protect our forests from current and future biological threats.

5 SUMMARY

5 POVZETEK

Podlubniki (Coleoptera: Curculionidae: Scolytinae) kot promotorji kroženja organske snovi igrajo ključno vlogo v gozdnih ekosistemih. Nekatere vrste lahko po izjemnih podnebnih dogodkih povzročijo tudi veliko okoljsko in gmotno škodo. Navadna smreka (*Picea abies*, (L.) H. Karst.) je ena izmed ekonomsko najbolj pomembnih drevesnih vrst v Evropi. Zaradi pretek-

lega sajenja na območja, manj primerna za njeno rast, je lahko dodatno dovzetna za izbruhe osmerozobega smrekovega lubadarja (*Ips typographus*, L.). Ob pojavu izbruhov te in drugih vrst škodljivih organizmov je identifikacija povzročitelja pomembna za načrtovanje omejitve izbruha in sanacije. Prav tako je ključnega pomena za zgodnjo detekcijo invazivnih tujerodnih vrst, kar omogoči zmanjšanje ali celo prepreči škodo, ki bi lahko nastala zaradi neomejenega širjenja na nova območja. Molekularne metode so tu odlična podpora tradicionalnim morfološkim identifikacijskim metodam, saj lahko omogočijo identifikacijo neodvisno od razvojnega stadija in celo na poškodovanih osebkih ali v sledovih.

Shranjevanje vzorcev pred molekularno analizo in izbira ekstrakcijskega postopka lahko vplivata na količino in kakovost pridobljene DNA. V raziskavi smo primerjali dva načina shranjevanja primerkov *I. typographus*, suho zamrzovanje in zamrzovanje v 96 % etanolu. V primerjavo ekstrakcijskih postopkov pa smo vključili štiri različne postopke na osnovi komercialno dostopnih ekstrakcijskih kitov dveh različnih proizvajalcev. P1 - Nucleospin DNA Insect Kit (Macherey Nagel, Düren, Nemčija); P2 - DNeasy Blood and Tissue Kit (Qiagen, Venlo, Nizozemska); P3 - Qiagen QIAamp DNA Micro Kit (Qiagen, Venlo, Nizozemska) brez nosilne RNA v pufru za vezavo; P4 - Qiagen QIAamp DNA Micro Kit (Qiagen, Venlo, Nizozemska) z nosilno RNA v pufru za vezavo. Ekstrakcije celokupne DNA smo z navedenimi kiti opravili po navodilih proizvajalcev. Z UV spektrofotometrom smo izmerili koncentracijo in izračunali končno količino pridobljene DNA, ki smo jo nato normalizirali glede na vhodno maso osebka, iz katere je bila pridobljena. Kvaliteto DNA in njeno uporabnost za molekularno identifikacijo pa smo ocenili s pomočjo polimerazne verižne reakcije in sekvenciranja po Sangerju z dvema različnima paroma začetnih oligonukleotidov. DNA smo ocenili kot uporabno za molekularno identifikacijo, če je v polimerazni verižni reakciji dala pomnožke, s pomočjo katerih smo s sekvenciranjem prišli do kromatogramov, ki so bili dovolj kakovostni za zanesljivo identifikacijo tarčnega osebka s pomočjo primerjave z referenčno sekvenco. DNA smo pridobili v 24 postopkih iz posameznih osebkov *I. typographus*, po 12 na postopek shranjevanja in po 3 na postopek ekstrakcije celokupne DNA. Iz statistične analize količine ekstrahirane DNA smo zaradi nosilne RNA v ekstraktu in narave kvantifikacije izločili protokol P4. Kljub temu, da se količina pridobljene DNA na gram vhodnega materiala med obema postopkoma shranjevanja ni statistično razlikovala, pa je bila razlika v uporabnosti DNA za PCR očitna iz vizualne ocene pomnožkov na gelu. Enaka vhodna količina DNA je namreč v isti polimerazni verižni reakciji dala na videz očitno bolj svetle pomnožke pri postopku shranjevanja v etanolu kot pri postopku shranjevanja brez etanola. Ekstrakti, pridobljeni iz osebkov, shranjenih v etanolu, so na gelu dali pomnožke z večjo intenziteto kot tisti, ki so bili rezultat suhega zamrzovanja. Razlika v kvaliteti DNA je bila lahko rezultat ohranitvene vloge etanola, ki v tkivih zamenja vodo in tako prepreči škodo, ki bi drugače nastala ob prisotnosti vode. Poleg tega pa etanol denaturira proteine, vključno s tistimi, ki imajo sposobnost razreza ali drugačnega vpliva na DNA. Statistična primerjava postopkov ekstrakcije celokupne DNA pa je pokazala, da se pridobljena količina DNA na gram vhodnega materiala statistično razlikuje le med protokoloma P2 in P3. Prvi preizkušeni par začetnih oligonukleotidov, LCO1490/HCO2198, se ni izkazal za uporabnega, saj smo uporabne sekvence pridobili le pri dveh od 24 ekstraktov, le-te pa so pripadale parazitskim ogorčicam iz reda Rhabditidae. Ta oligonukleotidni par je bil predhodno že uspešno uporabljen pri drugih vrstah podlubnikov, kar dodatno nakazuje na to, da je izbiro postopkov treba prilagoditi tarčni taksonomski enoti. Bolj uspešna je bila polimerazna verižna reakcija z začetnima oligonukleotidoma Ips_F_cons/Ips_R_cons, saj smo pri večini preizkušenih postopkov pridobili pomnožke pričakovane dolžine okoli 936 baznih parov. Kljub temu so bili za nadaljnje sekvenciranje uporabni le pomnožki, pridobljeni s protokoloma P1 in P2.

Med raziskavo smo potrdili, da lahko izbira postopkov shranjevanja in ekstrakcije celokupne DNA občutno vpliva na izid molekularnih postopkov za identifikacijo *I. typographus*. Izbor postopkov mora posledično sloneti na eksperimentalnih podatkih za načrtovani tip vzorcev in vrste molekularne analize. V naši raziskavi se je za optimalno izkazala uporaba kombinacije shranjevanja v etanolu in ekstrakcijskega postopka P1, pri katerem smo uporabili ekstrakcijski kit Nucleospin DNA Insect Kit nemškega proizvajalca Macherey Nagel. Ker molekularne metode pridobivajo pri pomembnosti v hitri identifikaciji že obstoječih in novo vnesenih vrst gozdnih škodljivcev, je naša raziskava eden izmed ključnih korakov pri zagotavljanju zanesljivosti rezultatov. To pa nam bo v prihodnosti omogočilo širjenje nabora orodij, s pomočjo katerih ščitimo naše gozdove pred obstoječimi in še prihajajočimi škodljivci.

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