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CTAB PROTOCOL OPTIMIZATION FOR HIGH-PURITY DNA EXTRACTION FROM ACIDIC SOIL IN THE HABITAT OF WILD BILBERRY (*VACCINIUM MYRTILLUS* L.)

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Two modified versions of the cetyltrimethylammonium bromide (CTAB)-based method were compared with a commercial kit to develop a cost-effective and efficient protocol for high-purity DNA extraction from acidic soil in the habitat of wild bilberry *Vaccinium myrtillus* L. The CTAB-NB (no bead treatment) approach resulted in poor outcomes in both yield and quality (1.56 and 0.37 at absorbance ratios of 260/280 and 260/230). The addition of bead treatment in 0.1 *mm* PowerBead Tubes (Qiagen), coupled with extended mixing (CTAB-B), increased DNA yield by more than eight times and substantially improved DNA purity, yielding 545.76 *ng* DNA per *g* of soil with 260/280 and 260/230 absorbance ratios of 1.87 and 1.62, respectively. The commercial kit provided high-yielded (856.08 *ng* DNA per *g* of soil) and pure DNA with a 260/280 ratio of 1.95 and a 260/230 ratio of 2.10. The CTAB-B protocol is cost-efficient and provides high-purity DNA suitable for metagenomic PCR amplification.

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Introduction. Soil is a major pool for microbial genetic diversity. Soil microbes play a key role in maintaining soil health and vital functions, influence soil fertility, phytopathogens control, plants stress tolerance and enhance nutrient use efficiency, particularly in low-fertility ecosystems, where symbionts help plants acquire limited resources [1, 2]. Analysis of soil microbiomes provides insights into the structure, functional networks, and roles of microbial communities in the sustainable development of ecosystems [1–3]. Recent studies of microbial communities have increasingly relied on metagenomic approaches [4]. As these methods rely on accurate genetic data, high-quality DNA extraction from soil samples is crucial for successful analysis. The extraction efficiency impacts not only DNA yield, purity, and integrity but also subsequent PCR reactions, potentially leading to biased results. Protocols must address challenges such as incomplete cell disruption

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and the presence of soil organic substances (e.g., fulvic and humic acids) that inhibit DNA polymerase activity and interfere with hybridization [5–9]. Evaluating these factors is essential to ensure the reliability and reproducibility of microbiome studies.

Soil is a challenging system for DNA extraction. Its matrix, shaped by silt, clay, sand, and organic matter content that organizes into micro- and macro-aggregates, hosts microorganisms unevenly distributed within microaggregates and macropores outside of them [10, 11]. Thus, protocol adjustments for studying soil microbial communities are necessary for each specific soil type.

Soil DNA extraction methods include mannitol, PEG/NaCl, and CTAB-based approaches, with various commercial kits available that differ in efficiency [5, 12, 13]. The extraction protocols typically include a cell lysis step, followed by DNA separation from impurities. Mechanical, physical and chemical treatments have been employed to improve cell lysis. For instance, shaking the sample in lysis buffers containing high concentrations of detergents, inclusion of enzymes or chaotropic agents, bead beating, sonication and freeze-thaw procedures all help to rupture cells and facilitate DNA release [6, 14, 15–17]. The yield and quality of extracted DNA are commonly measured using spectrophotometry. The accepted standards for DNA purity are a 260/280 absorption ratio between 1.8 and 2.0, and a 260/230 ratio between 2.0 and 2.2 for "pure" DNA. High-quality DNA should also demonstrate integrity, which is typically evaluated through visualization on an agarose gel [18, 19].

While several commercial kits are available for soil DNA extraction, their cost and availability can pose challenges for small research groups with limited funding. Additionally, some methods require specialized equipment, such as homogenizers, which may not be accessible to all researchers. Other costly facilities, like –80°C freezers or liquid nitrogen, which are used in some extraction protocols, can also be limited. Therefore, developing a cost-effective method/protocol for obtaining high-quality soil DNA with adequate yield and purity, using in-house approaches, is essential.

Bilberries (*Vaccinium myrtillus* L.) are valuable berries rich in anthocyanin and known for their numerous health-promoting properties [20]. *V. myrtillus* L. grows in Armenia at altitudes of 2300–2900 *m* above sea level, marking the southernmost edge of its global distribution. However, the bilberry ecosystem, including the associated microbial communities, remains largely unexplored. Studies on *Vaccinium* species have shown that soil microbiota composition varies with environmental conditions, affecting plant-microbe interactions and overall plant vitality and quality [21, 22]. Investigating the soil microbiomes associated with bilberry in Armenia is essential in the context of climate change, species expansion and resilience to environmental stresses. This research may strengthen conservation efforts, refine cultivation practices, and enhance the nutritional and medicinal properties of bilberries. In this paper, as part of our pioneering studies on the soil microbiomes associated with bilberries in Armenia, we compare DNA extraction protocols from *V. myrtillus* L. habitat soil, including a commercial kit and modified CTAB-based methods, to establish a cost-effective and reliable protocol for soil metagenomics studies.

Material and Methods.

Soil Sampling. The soil was collected from a habitat of *V. myrtillus* L. plants near the village of Hankavan, Kotayk Region, Armenia, at the altitude of 2450 *m* a.s.l., aliquoted and stored at –20℃ until further use. Soil chemical analysis was done at the Analytical Laboratory Qlab, Thessaloniki, Greece. The soil was classified as sandy loam; the acquired properties are listed in Tab. 1.

Soil DNA Extraction Methods. A soil sample was sieved through a 2 *mm* sieve twice before proceeding with the extraction to ensure uniform soil particle size for treatments. Two technical replicates were used for each extraction method.

DNA Extraction Using CTAB Method Without Beads (CTAB-NB). CTAB-based DNA extraction from soil samples was performed as described by Panosyan et al. [23] with some modifications. Briefly, 250 *mg* of soil sample was mixed with 1.5 *mL* of lysis buffer (400 *mM* NaCl, 750 *mM* sucrose, 20 *mM* EDTA, 50 *mM* Tris-HCl (pH 9)) in a 2 *mL* tube and incubated at room temperature for 3 *h* with occasional stirring. The mixture was frozen at –20℃ for 24 *h* and then incubated at 55℃ for 30 *min*. Six hundred μL of the resulting suspension was mixed with 100 μL of lysozyme solution (100 *mg*/*mL*) and incubated at 37℃ for 30 *min* with continuous shaking. After the addition of 1 μL RNase (10 *mg*/*mL*), 20 μL proteinase K (20 *mg*/*mL*), and 100 μL 10% sodium dodecyl sulfate (SDS), the suspension was incubated at 55℃ for 1 *h*, followed by mixing with 100 μL of 5 *M* NaCl solution. After 20 *min* of incubation at 65℃ with 80 μL of preheated 0.7 *M* CTAB/0.274 *M* NaCl mixture, 785 μL chloroform-isoamyl alcohol (24:1) was added, and the mixture was centrifuged at 15 000 *rpm* for 5 *min*. The obtained supernatant was precipitated with an equal volume of chilled isopropanol (at –20℃) and incubated at 4℃ for 1 *h*. The pellet was recovered by centrifugation at 15 000 *rpm* at 4℃, washed with 1 *mL* 70% ethanol, and dissolved in 20 μL TE buffer (Tris-HCl 10 *mM* (pH 7.8); EDTA 1 *mM* (pH 8)) after the final centrifugation at 15 000 *rpm* for 15 *min*.

DNA Extraction Using CTAB Method with Beads (CTAB-B). The extraction protocol was followed as described above with the addition of bead treatment. Instead of using 2 *mL* tubes, 250 *mg* of soil was placed into PowerBead Tubes (Qiagen, Germany) containing 0.1 *mm* glass beads and vortexed with 1.5 *mL* of lysis buffer for 30 *min* before a 3-hour incubation at room temperature. The protocol was then continued as outlined.

DNA Extraction Using DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germany). The extraction of DNA was performed according to the manufacturer's instructions. Briefly, 250 *mg* of a soil sample was placed into the PowerBead Tubes and gently vortexed to mix. Sixty μL of Solution C1 was added to the sample, and the slurry was homogenized in a Beadbug 3 microtube homogenizer ("Benchmark Scientific", USA) for 45 s at 4000 *rpm*. After 30 *s* of centrifugation at 10 000×g, the supernatant was transferred to a clean 2 *mL* tube, mixed with 250 μL of Solution C2, and incubated at 4℃ for 5 *min*. After a 1 *min* centrifugation at 10 000×g, 600 μL of the supernatant was transferred to a clean 2 *mL* tube and mixed with 200 μL of Solution C3, then incubated for another 5 *min* at 4℃. The 750 μL of the supernatant, collected after the centrifugation under the same conditions, was mixed with 1200 μL of Solution C4 and loaded onto an MB Spin Column in 675 μL

volumes, repeatedly, by centrifuging the tubes at 10 000×g for 1 *min*, and discarding the flow-through. The membrane was washed with 500 μL of Solution C5 using centrifugation for 30 *s* at 10 000×g and dried by subsequent centrifugation for 1 *min* at 10 000×g. The DNA was eluted with 50 μL of Solution C6 by 30 *s* centrifugation at 10 000×g. The extracted DNA was stored at –20℃ until further use.

Evaluation of DNA Yield and Purity. The DNA concentration of the soil sample was measured by using a DeNovix DS-11 spectrophotometer (DeNovix Inc., USA). The purity of the extracted DNA was determined by assessing the ratio of absorbance maxima at 260 *nm* and 280 *nm* (260/280) and the ratio of absorbance maxima at 260 *nm* and 230 *nm* (260/230).

PCR Amplification of Isolated Soil DNA. Soil DNA was amplified by PCR using an Arktik Thermal Cycler (Thermo Fisher Scientific, USA). Each 20 μL PCR mixture contained 20 *ng* of DNA template, 4 μL SolisFAST Master Mix (5×) (Solis BioDyne, Estonia), 0.5 μL of each forward and reverse primers (10 μM), and nuclease-free water up to 20 μL. The 16S rRNA region was amplified by using 16S rRNA primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The amplification cycle consisted of an initial denaturation step of 30 *s* at 94℃, followed by 35 cycles of 1 *min* at 94℃ (denaturation), 1 *min* at 55℃ (annealing), and 2 *min* at 72℃ (extension), with a final extension step for 5 *min* at 72℃.

Analysis of the Extracted DNA and PCR Products. To check the integrity of the extracted DNA and visualize the amplified PCR products, electrophoresis was performed on a 1% agarose gel in 1× TAE buffer, stained with ethidium bromide (0.5 $\mu g/mL$). The gels were analyzed using a Gel Doc EZ Imager Gel Documentation System (Bio-Rad, USA).

Results and Discussion. The physicochemical properties and macro- and micronutrient content of the soil sample collected from a habitat of *V. myrtillus* plants are presented in Tab. 1.

Table 1

Properties of the soil

Soil Physicochemical Properties		Soil Macronutrient and Micronutrient Content, *ppm*			
pH	5.4				
Conductivity, *mS*/*cm*	0.22	P	8.87	B	1.17
Salinity, psu	0.12	$N\text{-}NO_3$	33.06	Zn	1.46
Organic matter, %	5.76	Ca	3218	Fe	116
Clay, %	3.4	Mg	427	Mn	4.35
Silt, %	27.1	K	177	Na	142
Sand, %	69.6	Cu	1.61	CEC*	20.27

Note: * Cation Exchange Capacity, *meq*/100 *g.*

The results of community DNA extraction from a soil sample (Tab. 2, Fig. 1), comparing two modified CTAB-based protocols, CTAB-NB and CTAB-B, and a commercial kit (KIT). According to the obtained results, the commercial DNeasy PowerLyzer PowerSoil Kit provided a high yield of high-quality DNA with a 260/230 ratio of 2.1 and a 260/280 ratio of 1.95.

Table 2

Comparison of amount and purity of DNA from different extraction protocols: CTAB-method with no beads (CTAB-NB), CTAB-method with beads (CTAB-B), a commercial kit (KIT)

Extraction method	DNA concentration, *ng/μL*	Average DNA concentration, *ng/μL*	Average DNA yield, *ng/g* soil	260/280	Average 260/280	260/230	Average 260/230
CTAB-NB	15.92	16.15	64.6	1.59	1.56	0.36	0.37
	16.38			1.54		0.37	
CTAB-B	139.24	136.44	545.76	1.85	1.87	1.55	1.62
	133.63			1.89		1.69	
KIT	214.36	214.02	856.08	1.95	1.95	2.09	2.10
	213.69			1.96		2.10	

In contrast, DNA extracted using the CTAB-NB method resulted in poor outcomes in both yield and purity (Tab. 2 and Fig. 1). The 260/280 ratio of 1.56 and the 260/230 ratio of 0.37 indicate a high level of impurities, while the absence of a band on the agarose gel suggests a low performance of the method in obtaining intact, high-molecular-weight DNA from the soil. The 260/280 ratio below 1.6 indicates the presence of proteins, phenol, or other contaminants that absorb strongly at or near 280 *nm*. The 260/230 ratio considerably lower than the expected purity standards of 2.0–2.2 may indicate lipids and carbohydrates, as well as guanidine HCl, EDTA, salts, or phenol that absorb at 230 *nm*, which may be introduced by extraction procedures [18]. High concentrations of humic substances can compromise the quality of extracted DNA, requiring additional purification steps [9, 24]. Due to their similar charge and size properties, humic acids may interfere with DNA measurements, as they absorb at both 280 *nm* and 260 *nm*, potentially affecting the spectrophotometric quantification and quality assessments [9]. To ensure accurate DNA quantification, fluorometric measurements are recommended alongside spectrophotometric readings [25]. Humic acids have been reported to generate considerable drawbacks with downstream applications of DNA, involving interference in DNA polymerase activity in PCR reactions [24, 26]. Thus, the freeze-thaw cell lysis approach, with freezing at –20°C instead of the recommended –80°C and shaking the soil slurry with lysis buffer alone, was ineffective in disrupting cells, leading to poor DNA quality. Yet, freezing at –20°C is effective for DNA extraction from sludge samples [27]. The combination of freeze-thaw with chemical and enzymatic extraction was shown to be superior to ones without the freeze-thaw step [15].

The addition of bead treatment to the protocol (CTAB-B) significantly improved the extraction results, increasing the yield of the extracted DNA by more than eight times and achieving high purity standards, with a 260/280 ratio of 1.87 (Tab. 2). The DNA was intact and free from RNA contamination (Fig. 1). The 260/230 ratio of 1.62 indicates some contamination; however, the level of impurities, according to the data, is unlikely to interfere with downstream applications. The obtained purity results surpass those of many commercial kits and in-house protocols [28]. The incorporation of the bead-treatment step has been reported to enhance sample homogenization and lysis, the breakdown of the lipid bilayer of cell membranes and promote efficient penetration of lysis reagents into cellular compartments, facilitating the release of DNA [5, 6, 29]. In the CTAB-B and

KIT methods, the diameter of the used glass beads (PowerBead Tubes) was 0.1 *mm*. However, the diameter and material of the grinding beads may vary in different kits and protocols and need to be optimized for a specific soil material [29]. The PowerBead Tubes, available commercially from Qiagen, are around half the price of the kit. Thus, the CTAB-B method can serve as a low-cost alternative to accessing metagenomic content from the soil sample.

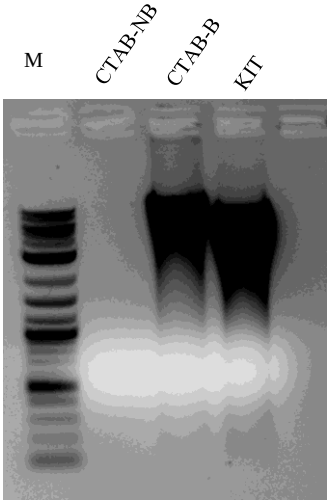

Fig. 1. Yield and purity of the extracted DNA. Visualization of 2 μL DNA extracted with three different protocols on 1% agarose gel:
CTAB-method without beads (CTAB-NB);
CTAB-method with bead treatment (CTAB-B);
extraction with a commercial kit (KIT);
M – GeneRuler 1 *kb* Plus DNA ladder.

The extracted DNA was proceeded further for PCR amplification. The 16S rRNA, commonly used for bacterial identification, was chosen as a marker for PCR. The results suggested that both the CTAB-B method and the commercial kit yield high-quality DNA suitable for the amplification (Fig. 2).

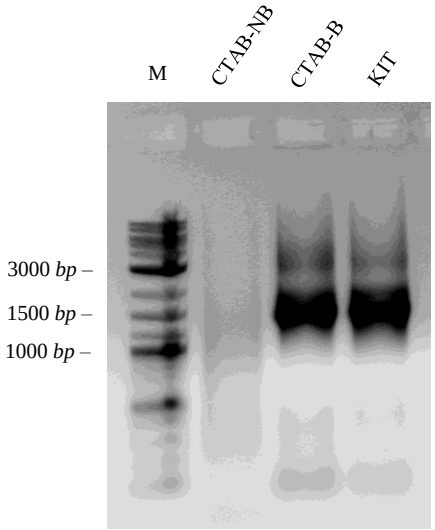

Fig. 2. Visualization of the PCR amplification products of DNA, isolated by three methods. The 16S rRNA full-length region was amplified using 20 *ng* of soil DNA templates, isolated by CTAB-NB, CTAB-B, and KIT methods; M marks a 1 *kb* DNA ladder. The electrophoresis was performed on 1% agarose gel.

Both bands corresponded to the expected 16S rRNA amplified region of approximately 1500 *bp* and were similar in quantity. The absence of a band in the DNA template extracted using the CTAB-NB method confirmed that the obtained DNA was unsuitable for further PCR amplification. This is because DNA polymerase, required for amplification, needs contamination-free sites for proper functioning. The unsuccessful PCR may have been caused by DNA-adhering substances such as humic and fulvic acids, which can negatively affect DNA hybridization efficiency.

The amplification results indicated that both the CTAB-B and commercial kit methods were effective for producing DNA free from PCR inhibitors and reproducible for obtaining amplified genetic regions of sufficient quality and integrity, which may provide comprehensive information on microbial biota [5, 24]. Further metagenome sequencing of the amplicons will clarify whether the extraction method influenced the microbiome structural composition data.

Conclusion. The comparison of three soil DNA extraction methods revealed that the CTAB-NB method yielded poor-quality DNA with poor purity and insufficient quantity for downstream applications. In contrast, the CTAB-B method, which incorporated bead treatment, significantly improved DNA yield and purity. While the commercial kit provided the best results, the CTAB-B method generated sufficient DNA for metagenomic amplification offering a cost-effective alternative for laboratories with limited resources. This protocol can support soil microbiome studies, agricultural research, and environmental monitoring, where high-quality DNA extraction is crucial. However, further optimization may be required for soils with high organic matter content and/or extreme compositions. Future investigation will focus on refining this method and evaluating its performance across a broader range of soil samples.

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ՎԱՅՐԻ ՀԱՊԱԼԱՍԻ (*VACCINIUM MYRTILLUS* L.) ԾՐՋԱԿԱ ԹԹՎԱՅԻՆ ՀՈՂԵՐԻՑ ՑԵՏԻԼԵՌՄԵԹԻԼԱՄՈՆԻՈՒՄ ԲՐՈՄԻԴԻ ՀԻՄՔՈՎ ԲԱՐՁՐ ՄԱՔՐՈՒԹՅԱՄԲ ԴՆԹ-Ի ԱՆՋԱՏՄԱՆ ԸՆԹԱՑԱԿԱՐԳԻ ԼԱՎԱՐԿՈՒՄ

Ցետիլեռմեթիլամոնիում բրոմիդի (CTAB) հիմքով մեթոդի երկու ձևափոխված տարբերակներ համեմատվել են առևտրային հավաքածուի հետ վայրի հապալասի (*Vaccinium myrtillus* L.) շրջակա թթվային հողերից բարձր մաքրության ԴՆԹ-ի անջատման մատչելի և արդյունավետ ընթացակարգ մշակելու նպատակով: Պարզվել է, որ CTAB-NB (առանց գնդիկային մշակման) ընթացակարգի կիրառմամբ անջատված ԴՆԹ-ի թե ելքը, և թե որակը (1,56 և 0,37 արժեքներ՝ համապատասխանաբար 260/280 և 260/230 ալիքներ կլանման հարաբերակցություններում) զգալի ցածր է եղել: Ցույց է տրվել, որ 0.1 *մմ* տրմագծով գնդիկներ պարունակող PowerBead (Qiagen) սրվակներում նմուշի մշակումը՝ զուգորդված երկարատև խառնումով (CTAB-B ընթացակարգ), ԴՆԹ-ի ելքը մեծացնում է ութ անգամ՝ հասցնելով 545.76 *նգ*-ի՝ 1 *գ* հողի հաշվարկով, իսկ մաքրությունը զգալիորեն բարելավում է՝ գրանցելով 1,87 և 1,62 արժեքներ՝ համապատասխանաբար 260/280 և 260/230 ալիքների կլանման հարաբերակցություններում: Առևտրային հավաքածուն ապահովում է ԴՆԹ-ի ավելի բարձր ելք (856,08 *նգ* ԴՆԹ 1 *գ* հողի հաշվարկով) և մաքրություն՝

1,95 և 2,10 արժեքներով՝ համապատասխանաբար 260/280 և 260/230 ալիքի կլանման հարաբերակցություններում: Ցույց է տրվել, որ առևտրային հավաքածուի համեմատ CTAB-B ընթացակարգով ԴՆԹ-ի անջատումը հողից ավելի ծախսարդյունավետ է, և ապահովում է մետագենոմային հետազոտություններում պոլիմերազային շղթայական ռեկցիայով ամպլիֆիկացման համար անհրաժեշտ բարձր մաքրությամբ ԴՆԹ-ի ստացում:

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ОПТИМИЗАЦИЯ ПРОТОКОЛА ИЗВЛЕЧЕНИЯ ВЫСОКОКАЧЕСТВЕННОЙ ДНК С ИСПОЛЬЗОВАНИЕМ БРОМИДА ЦЕТИЛТРИМЕТИЛАММОНИЯ ИЗ КИСЛЫХ ПОЧВ МЕСТ ПРОИЗРАСТАНИЯ ДИКОЙ ЧЕРНИКИ (*VACCINIUM MYRTILLUS* L.)

Два модифицированных метода с использованием бромида цетилтриметиламмония (CTAB) были сравнены с коммерческим набором с целью разработки экономичного и эффективного протокола извлечения высококачественной ДНК из кислых почв в местах произрастания дикой черники (*Vaccinium myrtillus* L.). Метод CTAB-NB (без гомогенизации бисером) привел к низкому выходу и низкому качеству ДНК (отношения оптических плотностей 260/280 и 260/230 равны 1,56 и 0,37 соответственно). Обработка образца почвы 0,1 *мм* стеклянным бисером с использованием пробирок PowerBead от Qiagen (метод CTAB-B) увеличила выход ДНК более чем в восемь раз, достигнув 545,76 *нг* ДНК на *г* почвы, и существенно улучшила качество ДНК – 1,87 и 1,62 для 260/280 и 260/230 соответственно. Коммерческий набор обеспечил высокую продуктивность (856,08 *нг* ДНК на *г* почвы) и высокую степень чистоты ДНК – отношения 260/280 и 260/230 равны 1,95 и 2,10 соответственно). Метод CTAB-B является более экономичным и позволяет получать высококачественную ДНК, пригодную для проведения полимеразной цепной реакции для метагеномной амплификации.