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DEVELOPMENT OF DNA ISOLATION METHODS TO STUDY DERMATOMYCOSIS

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ABSTRACT

Dermatophytes are keratinophilic fungi that infect hair, skin, nails, and feathers, classified into seven genera within the *Arthrodermataceae* family. Species identification has evolved with molecular methods, leading to the adoption of the «One Fungus = One Name» system. Dermatophytes are categorized into anthropophiles, zoophiles, and geophiles, with human-to-human transmission typically involving Trichophyton species and animal-to-human infections linked to Microsporum. Accurate DNA extraction is essential for effective molecular detection, yet fungal cell walls present a significant challenge. This study evaluated three DNA extraction protocols to optimize fungal DNA isolation. Spectrophotometric analysis demonstrated that Protocol 3 yielded the highest DNA concentration (93.61–2008.9 ng/µL) with optimal purity (A260/280: 1.8–2.2), whereas Protocol 1 had lower efficiency. While Protocol 2 showed variable results, it provided better purity for some hosts. These findings underscore the need for optimized extraction methods to enhance dermatophyte DNA detection in clinical and veterinary diagnostics.

Keywords: Dermatophytes, fungus, DNA extraction, farm animals, *Trichophyton*, *Microsporum*, molecular analysis

INTRODUCTION

Dermatophytes are a type of fungus that invade and break down keratinized tissues such as hair, skin, nails, and feathers. These fungi are classified within the Ascomycota phylum, Eurotiomycetes class, Onygenales order, and the Arthrodermataceae family. Currently, there are seven recognised genera of dermatophytes: *Trichophyton, Epidermophyton, Nannizzia, Paraphyton, Lophophyton, Microsporum*, and *Arthroderma*. As with other families of fungi, the names of species have evolved over time as mycology has shifted from naming based on morphology and clinical presentations to incorporating molecular methods [1, 2]. Furthermore, the sexual form (teleomorph) and the asexual form (anamorph) were previously categorised as two distinct species with different names. Recently, the names for teleomorphs and anamorphs have been unified, leading to the implementation of the "One Fungus = One Name" system for the identification of species [3].

Dermatophytes are generally categorized into three groups based on their living environment: anthropophiles (which thrive on humans), zoophiles (which inhabit animals), and geophiles (which reside in the soil) [4, 5]. The lines between these categories can be unclear, as species may adapt to specific hosts and change their preferred environments [5]. Understanding these group classifications is crucial because the type of dermatophyte responsible for an infection can affect how it presents clinically [6, 7, 8]. There are over 40 species across all three categories that can potentially infect humans. The subsequent sections will explore habitat classification groups and the types of infections caused by the most clinically significant species [9, 10, 11].

Fungal DNA extraction presents challenges due to the rigid structure of fungal cell walls composed of chitin, glucans, and proteins. Commonly used approaches include, for example, mechanical disruption (e.g., bead beating, grinding in liquid nitrogen) – highly effective in breaking tough fungal cell walls but may shear DNA, it requires specialized equipment [12, 13], enzymatic digestion (e.g., lyticase, zymolyase) – facilitates gentle cell lysis with minimal DNA fragmentation; however, the cost of enzymes and incomplete digestion in some strains are limitations [14]. Chemical lysis with detergents (e.g., CTAB, SDS) – commonly used in combination with organic extraction (phenol-chloroform), providing good yield and purity, but involves hazardous chemicals and multiple steps [15]. Commercial kits – offer standardized protocols and convenience, though they can be expensive and sometimes yield lower DNA concentrations, especially with dermatophytes [16].

In our study, we modified the classic phenol-chloroform method with CTAB buffer and Proteinase K, allowing enhanced DNA yield and purity. This method balances efficiency and cost, making it suitable for routine use in clinical and veterinary laboratories.

MATERIALS AND METHODS

Sample collection

Biomaterial sampling from farm animals – cattle, sheep, and horses – was conducted across various farms to capture diverse genetic and health backgrounds. Samples were collected using standardised procedures and securely transported to the laboratory to preserve their integrity. Similarly, biological material from human subjects was collected in partnership with medical institutions, adhering to strict ethical standards. Informed consent was obtained from all participants, ensuring confidentiality and respect for their rights throughout the research process. Work with animal and human samples was carried out in the Research Platform for Agricultural Biotechnology (RPAB) and was approved by the Animal Ethics Committee of the S. Seifullin Kazakh Agrotechnical Research University (extract from protocol No. 2 dated October 3, 2022).

Primary Isolation of Culture

Biomaterials measuring 2-4 mm were placed in sterile Petri dishes following aseptic techniques, using Sabouraud agar supplemented with chloralimphenicol to inhibit the growth of

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bacterial flora. The samples were incubated at 28°C for a duration of 14-18 days.

Cultural and Morphological Identification of Fungi

Colonies of fungi were identified by observing the characteristics of aerial and substrate mycelium, as well as the morphology of hyphae, microconidia, and macroconidia, utilizing the «Identifier of Pathogenic and Opportunistic Fungi» by Sutton et. all, 2001 [17].

DNA extraction

DNA extractions were conducted utilising a modified standard phenol-chloroform method. To enhance DNA yield, the work-up was optimised during the primary processing of cultures.

Protocols for DNA Extraction:

A small piece of fungus (0.1 – 0.3 g) was transferred from the nutrient substrate to a mortar. To this, 300 µl of extraction buffer (composed of 2M Tris HCl at pH 8.0 (Thermo Fisher Scientific, USA), 0.5M EDTA (Sigma-Aldrich, Germany), NaCl, and 2% Cetyltrimethylammonium Bromide (CTAB) (BioChemica, A0805, Italy)) and Proteinase K (20 mg/mL) (Thermo Fisher Scientific, USA) were added. The mixture was then incubated for 14-16 hours at 65°C. DNA extraction proceeded using the standard phenol-chloroform (Thermo Fisher Scientific, 15593049, USA) method [18].

Biomass production was carried out in liquid Sabouraud nutrient medium, consisting of 4 g of glucose and 1 g of peptone, supplemented with 0.9 g of NaCl per 100 ml of distilled water. Aeration was provided by an orbital shaker (Biosan ES-20, Latvia) operating at a speed of 120-130 rpm. The biomass was cultivated for a duration of 3 to 6 days, until small globules formed, depending on the growth rate of the cultures. Globules, approximately 0.5-1.5 grams, were then separated from the substrate by centrifuging (3500-4000 rpm for 5-7 minutes) and transferred to buffers. To each mortar, 300 µl of the extraction buffer (comprising 2M Tris HCl at pH 8.0, 0.5M EDTA, NaCl and 0.8% CTAB) was added, along with Proteinase K (20 mg/mL). The mixture was incubated for 14 to 16 hours at 65 °C, after which DNA extraction continued using the standard phenol-chloroform method.

Biomass production was conducted in a liquid Sabouraud nutrient medium, with the same compositions and conditions. These globules were then separated from the substrate by centrifuging (3500-4000 rpm for 5-7 minutes) and approximately 0.5-1.5 g of globules transferred to extraction buffer with adding 300 µl of a 2% CTAB solution (comprising 2% CTAB, 1.4 M NaCl, 20 mM EDTA, and 500 mM Tris-HCl, brought to a final volume of 50 ml with distilled water) along with Proteinase K (20 mg/mL), the mixture was incubated at 65°C for 2 to 4 hours. DNA extraction was subsequently performed using the standard phenol-chloroform method.

Quantitative and qualitative analysis.

A comprehensive quantitative and qualitative analysis of the isolated DNA was conducted utilising optical spectrophotometry with a Nanodrop 2000 instrument. The quantification process involved measuring absorbance at specific wavelengths to determine DNA concentration and purity ratios (A260/A280 and A260/A230).

PCR analysis, electrophoresis and sequencing

A polymerase chain reaction (PCR) was performed using the ITS 1/4 (internal transcribed spacers) for differentiating species by sequencing. PCR was performed in a 25 µl reaction mixture containing 10× Taq buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 1 U Taq DNA polymerase and 200 µM dNTPs (Thermo Scientific, Carlsbad, California, USA), 10 pmol of each primer and 20 ng of extracted gDNA as a template. DNA segments were amplified using thermal cycling reactions for 35 cycles with primary denaturation at 94 °C for 5 min, followed by denaturation (94 °C for 30 s), annealing (55 °C for 45 s) and extension (72 °C for 50 s), with final extension at 72 °C for 5 minutes. Agarose gels (1.5%) were prepared in 1× TAE solution with 8 ng/µL ethidium bromide (Sigma, E1510). Electrophoresis was performed using 10 µL PCR products with a DirectLoad 100 bp Low ladder ready-to-use (Sigma, D3687-1VL) for 50 min at 120 V. The PCR-amplified target gene fragment was purified using a QIAquick PCR Purification Kit, (QIAGEN, Germany, Cat.: 28106), following the manufacturer's protocols. Sequencing was performed according to the manual for the Seq Studio Genetic Analyser (Thermo Fisher Scientific Applied Biosystems). The resulting nucleotide sequences were visually checked by the Bioedit program version 7.0. The nucleotide sequences of the studied species were compared with other sequences in the NCBI gene bank database by using the BLAST options (http://blast.ncbi.nlm.nih.gov).

RESULTS

Following the results from our development of the DNA extraction protocol, we performed an analysis that demonstrated the effectiveness of the third protocol. This improvement can be attributed to modifications made to the extraction buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, and 500 mM Tris-HCl), enabling us to achieve a substantial yield of DNA. The results of the qualitative and quantitative analysis are presented in Table 1.

Table 1. Qualitative and quantitative analysis of extracted DNA

№	Isolates	Dermatophytes species	1-protocol DNA concentration ng/µ	A260/280	2-protocol DNA concentration ng/µ	A260/280	3-protocol DNA concentration ng/µ	A260/280
1	Human-1	*Trichophyton spp.*	0.7	1.0	581.80	1.5	2033.2	1.8
2	Human-2	*Trichophyton spp.*	15.3	0.8	829.94	1.4	823.1	1.7
3	Human-3	*Trichophyton spp.*	14.9	1.3	53.624	1.4	336.1	1.8

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4	Cattle-1	Trichophyton spp.	45.7	1.6	776.46	1.4	1066.7	2.2
5	Cattle-2	*Trichophyton spp.*	23.5	1.4	55.098	1.1	284.10	2.0
6	Cattle-3	*Trichophyton spp.*	10.4	1.2	1268.87	1.3	193.28	2.1
7	Ovis-1	*Trichophyton spp.*	117.5	1.6	368.76	1.3	836.97	1.9
8	Ovis-2	*Trichophyton spp.*	78.2	1.4	57.655	1.4	1800.4	2.3
9	Ovis-3	*Trichophyton spp.*	63.8	1.4	625.73	1.4	789.4	2.2
10	Horse-1	*Microsporum spp.*	150.4	1.6	123.11	1.6	296.13	1.8
11	Horse-2	*Microsporum spp.*	174.0	1.6	312.00	1.6	2008.9	1.9
12	Horse-3	*Microsporum spp.*	207.1	1.3	98.013	1.0	118.316	2.0
13	Ovis-4	*Microsporum spp.*	340.5	2.8	272.047	1.8	93.61	1.8
14	Ovis-5	*Microsporum spp.*	180.7	3.2	62.615	1.3	146.778	1.8
15	Ovis-6	*Microsporum spp.*	94.2	2.5	42.338	1.4	361.79	2.2
16	Human-1	*Microsporum spp.*	139.6	0.9	27.040	1.4	458.88	1.9
17	Human-2	*Microsporum spp.*	48.3	1.0	1564.3	1.3	312.74	2.0
18	Human-3	*Microsporum spp.*	65.0	1.2	240.51	1.6	718.76	2.1

Table 2. DNA Concentration (ng/µL) Across Protocols

Observation	Protocol 1	Protocol 2	Protocol 3
Highest DNA Yield	Horse-3 (207.1 ng/µ)	Human-2 (1564.3 ng/µ)	Ovis-2 (1800.4 ng/µ)
Lowest DNA Yield	Human-1 (0.7 ng/µ)	Human-3 (27.04 ng/µ)	Horse-3 (118.3 ng/µ)
General Trend	Moderate DNA yields across species	Some isolates had significantly higher yields	Highest yields observed in certain samples

Table 3. DNA Purity (A260/280 Ratio) Comparison

Observation	Protocol 1	Protocol 2	Protocol 3
Highest Purity	None of the samples	1.8	1.8 – 2.2
Lowest Purity	0.8	1.0	1.7
General Trend	Ratios mostly around 0.8 – 1.6	Ratios mostly around 1.0 – 1.6	Higher purity values, but inconsistent

Quantitative analysis of the extracted DNA using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) revealed concentrations ranging from 429.2 to 2258.2 ng/µl, with notable variation across protocols and samples – some exceeding 2000 ng/µl. The A260/280 absorbance ratios, indicating potential protein or RNA contamination, also varied widely, generally falling between 1.0 and 2.8

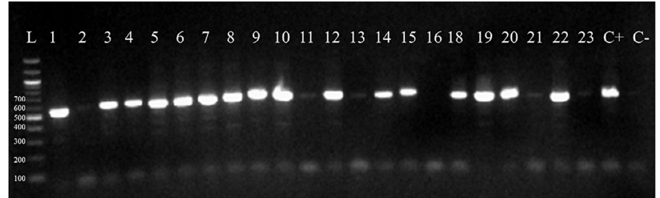
Protocol 3 generally resulted in the highest DNA concentrations, particularly for sheep and horse isolates. Protocol 2 also provided high yields but showed more variability. Protocol 1 yielded lower DNA concentrations in most cases.

Extreme A260/280 values in some samples indicate that the fungal cell wall was not completely destroyed, and some protein components and polysaccharides remained in solution. This also indicates the need for RNase treatment. Protocol 1 resulted in generally low purity values, indicating potential protein and RNA contamination. Protocol 2 had highly variable purity ratios, suggesting inconsistency in removing contaminants. Protocol 3 showed the highest A260/280 values in some cases, but also had inconsistencies.

Protocol 3 is the most effective for high DNA yields across all host types. For purity, Protocol 3 performs better for sheep, but Protocol 2 is better for human and horse samples.

Overall Summary: Protocol 1: Lower DNA yield, generally lower purity. Protocol 2: Higher variability in both DNA yield and purity. Protocol 3: Best for DNA concentration, but purity is inconsistent. Recommendation: Protocol 3 is preferred for DNA extraction if high yield is the priority, while Protocol 2 may be better for purity in some cases.

To validate the DNA extraction protocol, a PCR analysis was conducted on the marker regions of ITS 1/4. Figure 1 displays the results of the PCR analysis via electrophoresis.



L – 100 bp ladder, from 1 to 12 – *Trichophyton* spp. samples, from 13 to 23 – *Microsporum* spp.

Figure 1. Electrophoresis results of ITS 1/4 gene on dermatomycosis samples

Nucleic acid electrophoresis was performed to evaluate the quality of the isolated DNA samples. Figure 2 illustrates the electrophoresis results, which highlight the integrity and purity of the DNA extracted using three protocols.

The bands observed on the gel indicate not only the size of the DNA fragments but also the absence of RNA contamination and degradation. This also showcases that the third method (Figure 2C) employed was effective in yielding high-quality DNA suitable for downstream applications.

The obtained amplicons were subjected to sequencing analysis. As a result, nucleotide sequences of the species *Microsporum canis* (PX129058, PQ844828, PQ844832,

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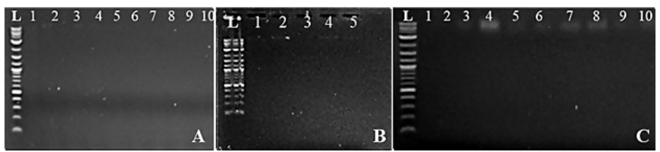

A: Protocol – 1; lane 1-10 DNA samples; B: Protocol – 2; lane 1-5 DNA samples; C: Protocol – 3; lane 1-10 DNA samples; L: DNA ladder 1 kb

Figure 2. Gel electrophoresis of genomic DNA samples for quality assessment.

PQ844833) and *Trichophyton mentagrophytes* (PQ844693, PQ844689, PQ844679) were obtained and deposited in the GenBank database.

DISCUSSION

Dermatophytosis remains a significant public health and veterinary problem worldwide, caused by fungi of the genera *Trichophyton*, *Microsporum*, and *Epidermophyton* [4, 5]. In agreement with earlier studies emphasising the diagnostic importance of reliable DNA isolation [12, 19], our findings highlight that the choice of extraction protocol directly influences both yield and purity of fungal DNA, which in turn affects downstream PCR-based diagnostics.

Previous works have shown that fungal DNA isolation is challenging due to the rigid chitin–glucan cell wall [12, 20]. Our comparative evaluation of three protocols confirmed these limitations: Protocol 1 produced insufficient yields, consistent with earlier observations that simple chemical lysis without adequate disruption often results in low DNA recovery [21]. By contrast, Protocol 2, which included stronger lysis steps, produced variable but higher DNA concentrations. This aligns with Muñoz-Cadavid et al. (2010), who also reported improved yields with enzymatic and detergent-based methods but noted inconsistency across fungal isolates.

The superior performance of Protocol 3, yielding up to 2008.9 ng/µl with optimal A260/280 ratios (1.8–2.2), is comparable to results obtained by Than et al. (2015) and Garg et al. (2009), who demonstrated that CTAB-based buffers combined with phenol–chloroform extraction significantly improve DNA quality for dermatophytes. Notably, our results also showed that Protocol 3 performed particularly well for *Trichophyton* isolates, whereas purity for some *Microsporum* samples was slightly variable. This observation is in line with the findings of Griffiths et al. (2006), who reported that species-specific cell wall differences can influence extraction efficiency, underlining the need to adapt protocols to the target organism.

The clinical and veterinary relevance of optimising extraction methods is underscored by the increasing use of molecular tools for species identification and antifungal resistance monitoring [7]. For instance, the detection of terbinafine-resistant *Trichophyton indotineae* in recent surveys emphasises the necessity of robust molecular workflows to avoid false negatives caused by poor DNA quality. Our work supports this view, as Protocol 3 provided DNA of sufficient quantity and purity to amplify ITS markers and generate high-quality sequences successfully, consistent with earlier molecular phylogenetic studies on dermatophytes 8, 9, 10, 22].

Thus, our study contributes to the comparative body of work showing that while no universal protocol ensures equally high yields across all dermatophyte species, CTAB-based modifications of the phenol-chloroform method strike the best balance between yield, purity, cost, and reproducibility. These findings support the use of Protocol 3 in routine diagnostic laboratories, especially in regions where dermatophytosis in humans and livestock is endemic.

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CONFLICT OF INTEREST

The authors of the article have no conflict of interest.

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АННОТАЦИЯ

Дерматофиты – это кератинофильные грибы, которые поражают волосы, кожу, ногти и перья, классифицируемые по семи родам в пределах семейства *Arthrodermataceae*. Идентификация видов развивалась с помощью молекулярных методов, что привело к принятию системы «Один гриб = одно название». Дерматофиты подразделяются на антропофилов, зоофилов и геофилов, при этом передача от человека к человеку обычно включает виды *Trichophyton*, а инфекции от животных к человеку связаны с *Microsporum*. Точная экстракция ДНК необходима для эффективного молекулярного обнаружения, однако стенки клеток грибов представляют собой значительную проблему. В этом исследовании оценивались три протокола экстракции для оптимизации выделения ДНК грибов. Спектрофотометрический анализ показал, что протокол 3 дал самую высокую концентрацию ДНК (93,61–2008,9 нг/мкл) с оптимальной чистотой (A260/280: 1,8–2,2), тогда как протокол 1 имел более низкую эффективность. Хотя протокол 2 показал переменные результаты, обеспечил высокую чистоту для некоторых образцов. Эти результаты подчеркивают необходимость оптимизированных методов экстракции для улучшения обнаружения ДНК дерматофитов в клинической и ветеринарной диагностике. **Ключевые слова**: дерматофиты, грибок, экстракция ДНК, сельскохозяйственные животные, *Trichophyton*, *Microsporum*, молекулярный анализ

АННОТАЦИЯ

Дерматофиттер – шаш, тері, тырнақ және қауырсындарды зақымдайтын кератинофилді саңырауқұлақтар, *Arthrodermataceae* тұқымдасының жеті тұқымдастығына жіктеледі. Түрлерді анықтау молекулярлық әдістерді қолдану арқылы дамып, «Бір саңырауқұлақ = бір атау» жүйесін қабылдауға әкелді. Дерматофиттер антропофильді, зоофилді және геофильді болып жіктеледі, адамнан адамға жұғады, әдетте *Trichophyton* түрлері және *Microsporum* – мен байланысты жануарлардан адамға инфекциялар жатады. ДНҚ-ның дәл экстракциясы тиімді молекулалық анықтау үшін өте маңызды, бірақ саңырауқұлақ жасушаларының қабырғалары айтарлықтай қиындық тудырады. Бұл зерттеу саңырауқұлақ ДНҚ оқшаулауын оңтайландыру үшін үш ДНҚ экстракция протоколын бағалады. Спектрофотометриялық талдау 3 хаттаманың оңтайлы тазалығымен (A260/280: 1,8–2,2) ең жоғары ДНҚ концентрациясын (93,61–2008,9 нг/мкл) бергенін, ал 1 хаттаманың тиімділігі төмен екенін көрсетті. 2-хаттама өзгермелі нәтижелерді көрсеткенімен, кейбір үлгілер үшін жақсырақ тазалықты қамтамасыз етті. Бұл нәтижелер клиникалық және ветеринариялық диагностикада дерматофит ДНҚ-сын анықтауды жақсарту үшін оңтайландырылған экстракция әдістерінің қажеттілігін көрсетеді.

Негізгі сөздер: дерматофиттер, саңырауқұлақтар, ДНҚ экстракциясы, ауылшаруашылық жануарлары, трихофитон, микроспорум, молекулалық талдау