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Effect of *in vitro* Incubation Time and Drying on Genomic DNA Extracted from Bovine Ear Tissue Samples

Tejas Gohil[1#], Sandeep Kumar Donthula[2#], Bhumika Parmar[1], Hardik Poojara[2], Pankaj Limbachiya[1], A Sudhakar[2]

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ABSTRACT

Background: Use of ear punch samples as a competent source of nucleic acids from bovines, gained momentum around two decades ago, as their collection is quite easy and can be transported with minimal quantities of preservatives at ambient conditions. However, the auricular cartilage makes them more resistant to lysis, resulting in poor genomic DNA yields. The objective of the current study was to investigate whether drying can enhance the gDNA yields from ear tissue samples as a few past experiments gave an unanticipated good gDNA yields from completely dried tissue samples.

Methods: Upon receipt, ear tissue samples of the cattle and buffaloes were kept at room temperature for different time intervals (*in vitro* incubation) and then dried at various temperatures prior to gDNA extraction, with commercially available gDNA extraction kits from two manufacturers (MN and QIAGEN). The extracted gDNA samples were assessed with spectrophotometry, flourometry and agarose gel electrophoresis for concentration, purity and integrity.

Result: gDNA yields were gradually increasing in parallel to *in vitro* incubation time till 250 days and after that there was a decline. Further, gDNA yields were significantly higher for ear tissue samples that were not dried prior to extraction, which did not comply with the past experimental observations. Fluorometry-based quantifications revealed that drying of ear tissue samples at >50°C temperatures reduced double-stranded DNA, however up to 50°C the gDNA yield increased, indicating the tissue samples can be safely transported up to 50°C in the TSUs.

Key words: *Cytochrome b,* Drying, gDNA extraction, Incubation, PCR-RFLP, Tissue sampling unit (TSU).

INTRODUCTION

Being the first element of the central dogma, DNA is the most crucial biomolecule used in the fields of life sciences research as well as in clinical applications. A plethora of protocols (solution-based and silica column-based) have already been worked upon for the extraction of gDNA with desired quantity and quality from a variety of biological materials. Although many commercial kits are available from various manufacturers, DNA extraction from solid biological materials, especially from the ear tissue samples of bovines is often challenging due to cartilaginous tissue (Anchordoquy and Molina, 2007). The cartilage makes such tissues resistant to mechanical disruption too. The commercially available gDNA extraction kits may provide good quality gDNA but sometimes quantity can be a limitation. Pre-drying the samples may be a good modification to achieve a better quantity of gDNA in mushrooms and other plant species (Wand *et al*., 2017). However, different studies have shown gDNA isolation from various animal tissue samples, gDNA isolation from ear tissue samples is explored quite less (Liu and Harada, 2013; Whitlock *et al*., 2008; Peñafiel *et al*., 2019).

Usually, buffaloes are highly sensitive to touch, difficult to restrain and locating jugular vein requires expertise (Sears *et al*., 1978). On the other hand, the collection of ear tissue samples using the Allflex tissue sampling unit (TSU) requires comparatively less expertise. Although tissue sample collection has good overall applicability, very few studies have been reported on its use and standardization, earlier.

[1]Genomics Lab, Sabarmati Ashram Gaushala, Anand-388 001, Gujarat, India.
[2]Animal Breeding Group, National Dairy Development Board, Anand-388 001, Gujarat, India.
[#]These authors contributed equally to this work.

Corresponding Author: A. Sudhakar, Animal Breeding Group, National Dairy Development Board, Anand-388 001, Gujarat, India. Email: sudhakar@nddb.coop

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The present study was undertaken based on two following observations after extracting gDNA from ear tissue samples: 1) TSUs that lack the preservative due to crack, loose lid or due to evaporation, the quantity of the extracted gDNA from such ear tissues was reasonably good as compared to the samples with preservative fluid, 2) gDNA yield increased steadily with increase in *in vitro* incubation period till 250 days, post 250 days the yield decreased. The objective of the current study was to examine the effect of drying along with *in vitro* incubation time on gDNA from ear tissue samples.

MATERIALS AND METHODS

Sample availability

A total of 240 TSUs of *Bos indicus* (Gir-cattle) and 20 TSUs of *Bubalus bubalis* (Murrah-buffalo), available at Bio-Banking facility, D-Lab campus, NDDB, Anand, Gujarat, India, were used for drying and gDNA extraction, whereas the already available concentration data for 1700 tissue samples were used for *in vitro* incubation study. This study used the bovine ear tissue samples already available through other projects, ethical clearance was not required.

In vitro incubation time and drying of samples

In vitro incubation time is defined as the duration (days) between receipt of TSUs at the laboratory and gDNA extraction from them. Upon receipt at the laboratory, all the ear tissue samples were not always immediately processed for gDNA extraction, some of the TSUs were kept at room temperature for various periods of time. Later on, the samples with different *in vitro* incubation periods were compared in context to the quantity of gDNA extracted from them.

The TSUs store ear tissue samples in a specially designed preservative that protects the DNA from nucleases of the cell. The experimental setup for the current study is shown in Table 1. All the 240 cattle ear tissue samples were weighed individually for their initial weight (Wt.-Wet weight) and all were transferred into 1.5 ml microcentrifuge tubes to process them further. Then samples were divided into 8 groups (MN_PD_30, MN_PD_40, MN_PD_50, MN_PD_60, Q_PD_60, MN_PD_RT, MN_ND and Q_ND) irrespective of their Wt. as shown in table 1. Out of 240 cattle samples, 130 samples belonging to MN_PD_30, MN_PD_40, MN_PD_50, MN_PD_60 and Q_PD_60 groups were dried at four different temperatures *i.e.* 30°C, 40°C, 50°C and 60° C respectively in a dry bath (Genetix, India) and 10 tissue samples were dried at room temperature. After drying, all 140 tissue samples were again weighed for their final weight (Wd - Dry weight) and the moisture lost from the tissue samples was calculated as follows.

$$\text{Moisture loss ratio} = \frac{Wt - Wd}{Wt} \times 100$$

All 140 dried cattle ear tissue samples and the remaining 100 cattle ear tissue samples along with 20 buffalo ear tissue samples were directly processed for the gDNA extraction.

Extraction of gDNA

The gDNA was extracted using Nucleospin Tissue kit (NTK) from Macherey-Nagel and QIAamp DNA Micro kit (DMK) from QIAGEN from all ear tissue samples as shown in Table 1 by following manufacturer's protocol.

gDNA quantity and quality check

The quantity and purity of the extracted gDNA samples were evaluated by measuring the following absorbance ratios; a) the ratio of 260 nm and 280 nm, which is the method of assessment to detect protein contamination in DNA preparations and b) the ratio of 260 nm and 230 nm, which detects contaminants like guanidine, that is commonly used in column-based DNA extraction methods (TECAN infinite F nano+ spectrophotometer - Switzerland). The value for 260/280 absorbance ratio around 1.8 is considered as the standard for pure DNA, whereas the standard value for 260/230 absorbance ratio is within the range of 2.0-2.2 for uncontaminated DNA (Wilfinger *et al*., 1997).

The second parameter to check the DNA integrity is the agarose gel electrophoresis. Twenty four representative gDNA samples were run on a 1% agarose gel containing 5 µg/ml ethidium bromide in the electrophoresis unit (Biotech R and D Laboratories, Yercaud, India) with 1X Tris Acetate EDTA (TAE) buffer and a constant voltage of 90 V for 60 minutes. The agarose gels were visualized under UV light (XR+ Gel documentation system - Bio-Rad Laboratories, USA).

As the current study employed incubating the tissue samples at higher temperatures *i.e*. 40°C, 50°C and 60°C, it might have denatured the dsDNA. To specifically measure the double-stranded DNA (dsDNA), gDNA samples were quantified using the Qubit 4 Fluorometer (Thermo Fisher Scientific, USA) (Quick *et al*., 2014). Spectrophotometric assessment measures double-stranded as well as ssDNA and even nucleotides too.

PCR amplification of *cytochrome b* gene and RFLP

PCR amplification is a method of choice to evaluate the DNA samples for further downstream applications like microarray genotyping, sequencing *etc*. One representative cattle gDNA sample per group along with buffalo were amplified for mitochondrial *Cytochrome b* gene in a thermal cycler (Proflex PCR, Thermo Fisher) (Abdel and Ahmed, 2007). The PCR program for the same was as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec and a final extension at 72°C for 10 min.

For species identification, PCR-amplified products of cattle and buffalo from previous step were digested using *TaqI* restriction endonuclease for restriction fragment length polymorphism (RFLP) analysis. The composition of reagent mixture for RFLP analysis was; amplified PCR product 10 µl, nuclease free water 18 µl, 10X *TaqI* buffer 2 µl and *TaqI* enzyme 1.5 µl. For RFLP analysis, samples were incubated at 65°C for 2 hours in thermal cycler.

Statistical analysis

The quantitative comparison of gDNA between groups was performed using the Mann-Whitney U test, whereas qualitative comparisons for the 260/280 nm absorbance ratio and 260/230 nm absorbance ratio were executed by applying One Way Analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Previous observations

After receipt at the laboratory, the TSUs were visually examined for the preservative before gDNA extraction. The

yield was 201.66+112.19 ng/µl for such 24 dried ear tissue samples. The gDNA yields from around 1700 ear tissues, incubated for various time durations, showed that the samples with 221 to 250 days of *in vitro* incubation gave maximum gDNA yields, whereas, beyond 250 days, the gDNA concentration decreases (Fig 1).

Sample wet and dry weights

The average wet weight of 200 tissue samples (MN_PD_60, MN_ND, Q_PD_60 and Q_ND) was 31.83±7.04 mg (mean+SE). Group-wise average wet weight, dry weight and moisture loss ratio for the samples are mentioned in Table 2. The data shows that the samples that were dried at 60°C temperature, lost more than 70% of moisture.

Comparison between groups for gDNA yield and purity

Table 3 shows that MN_PD_60 group samples gave comparatively less gDNA yield as compared to the MN_ND group, as the maximum yields were 196 and 918.1 ng/µl respectively making it statistically significant with Mann Whitney U-test. Similarly, for Q_ND group samples gave considerably more DNA yields as compared to the Q_PD_60 group samples, since the average total yields were 7.86 µg and 3.42 µg respectively ($p<0.05$).

The gDNA concentrations of groups MN_PD_30, MN_PD_40, MN_PD_50 and MN_PD_60 showed that the gDNA concentration increased gradually as drying temperature increased from 30°C to 50°C, but at 60°C, the concentrations decreased (Fig 2). This indicated if in case the TSUs encounter up to 50°C temperature during transportation, samples still can give better gDNA yields. Although 50°C to 60°C temperatures are rare in the Indian subcontinent, a report suggested that the surface temperatures were exceeding 60°C temperatures in North India in April and May 2022 (https://www.hindustantimes.com/india-news/surface-temp-tops-60-c-satellite-images-show-101651343166998.html). The mean yields between MN_PD_30 and MN_PD_60 as well as between MN_PD_50 and MN_PD_60 using the Mann-Whitney U test were statistically significant ($p<0.05$). The concentration of gDNA for the ear tissue samples extracted with NTK gave superior yields compared to DMK and was statistically significant too. The above results indicated that tissue samples received from the field and processed without drying gave better yields.

Further, the absorbance ratio at 260/280 nm was near 1.8 for all samples of all groups, indicating pure DNA preparations. The One Way ANOVA for 260/230 nm absorbance ratio revealed that the difference between MN_PD_60 and MN_ND as well as between Q_PD_60 and Q_ND was statistically significant ($p<0.05$).

The assessment of gDNA yields for spectrophotometric and fluorometric measurements are shown in Fig 3.

Table 1: Experimental setup of the study.

Group name	Species	No. of samples	Modification	Temperature for drying (°C)	DNA extraction kit used
MN_PD_30	Cattle	10	Pre-drying	30	NTK
MN_PD_40	Cattle	10	Pre-drying	40	NTK
MN_PD_50	Cattle	10	Pre-drying	50	NTK
MN_PD_60	Cattle	50	Pre-drying	60	NTK
Q_PD_60	Cattle	50	Pre-drying	60	DMK
MN_PD_RT	Cattle	10	Pre-drying	Room temperature	NTK
MN_ND	Cattle	50	No drying	NA	NTK
Q_ND	Cattle	50	No drying	NA	DMK
MN_ND	Buffalo	20	No drying	NA	NTK

NA- Not applicable.

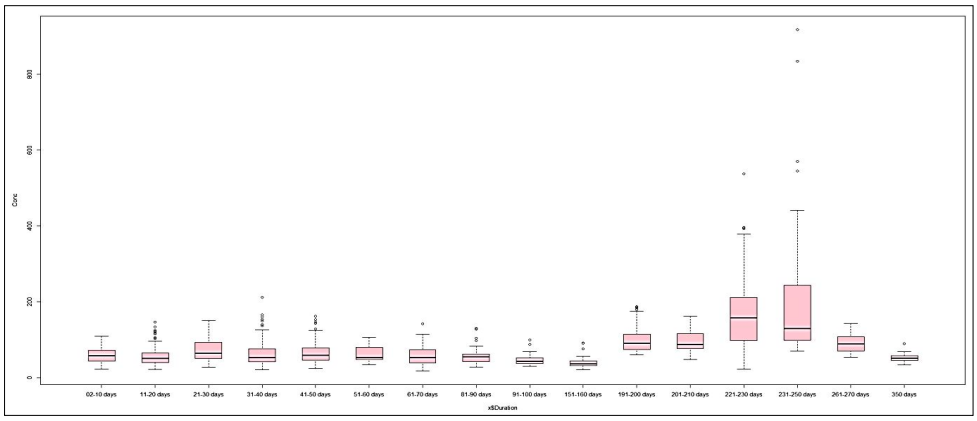


Fig 1: Effect of *in vitro* incubation time on quantity of gDNA extracted from ear tissue samples.

Fig 3 reveals that when samples were dried at 60°C, spectrophotometric measurements outweigh the fluorometric measurements and when samples were not dried, fluorometric measurements exceeded the spectrophotometric measurements.

Agarose gel electrophoresis of gDNA samples

The agarose gel image showed that nearly all the samples from all four groups showed intact DNA bands on the agarose gel (Fig 4). The largest band of the DNA ladder is ~21 kb, which means the size of the gDNA fragments for all the samples fall near 21 kb.

Table 2: Average wet weight, dry weight and moisture loss ratio of tissue samples.

Group	Average wet weight (mg) (Mean±SE)	Average dry weight (mg) (Mean±SE)	Moisture loss ratio (%)
MN_PD_60	35.36±0.85	9.85±0.38	72.36±0.57
MN_ND	29.04±0.70	NA	NA
Q_PD_60	31.37±1.11	8.37±0.38	73.38±0.59
Q_ND	31.53±1.10	NA	NA
MN_PD_30	34.96±3.10	19.81±2.27	43.82±2.61
MN_PD_40	36.58±1.61	13.78±1.11	62.5±2.04
MN_PD_50	36.24±5.53	15.42±3.33	63.16±4.49

NA- Not applicable.

PCR and RFLP

PCR amplification of the *cytochrome b* gene was successful for randomly selected gDNA samples of all groups, with a product size around 359 bp. This gene is widely used for animal species identification due to variability in its sequence (Kumar *et al*., 2022; Jain *et al*., 2007). Species identification using PCR-RFLP is performed to identify reasons for genotyping failure and remedial actions. RFLP of the cattle gDNA did not generated any fragments, whereas two DNA fragments of 191 bp and 168 bp were seen for buffalo (Fig 5).

The objective of the current study was to maximize the total yield of gDNA from the ear tissue of cattle and buffalo. The anatomical structure of the ear pinnae of bovines showed the presence of auricular cartilage surrounded by skin on the abaxial and axial sides Rashid *et al*. (1987), functioning to provide the framework for the organ due to its tough, flexible and elastic nature. The type of cartilage found in the ear pinna is the elastin cartilage, composed of chondrocytes and surrounding dense extracellular matrix. The number of cells found in cartilage is low as compared to the normal epidermis of the body (Parvizi and Kim, 2010). The above facts lead to

Table 3: Summary statistics of the spectrophotometric measurements (Concentration (ng/µl, total DNA yield (µg), absorbance ratio at 260 nm/280 nm and 260 nm/230 nm).

Group (Sample size)	Conc. (ng/µl) Mean±SE	Total yield (µg) Mean±SE	260/280 ratio Mean±SE	260/230 ratio Mean±SE
MN_PD_60 (50)	61.722±4.59*	3.7±0.27	1.90±0.0	2.65*±0.04
MN_ND (50)	302.95±21.71*	18.177±1.30	1.89±0.0	2.34*±0.0
Q_PD_60 (50)	57.052±3.69#	3.42±0.0	1.86±0.0	1.44#±0.04
Q_ND (50)	131.034±10.00#	7.86±0.60	1.87±0.00	1.66# ±0.06
MN_PD_30 (10)	113.74±13.26	6.82±0.79	1.89±0.0	2.37±0.03
MN_PD_40 (10)	128.21±11.98	7.7±0.72	1.87±0.0	2.25±0.01
MN_PD_50 (10)	163.33±29.08	9.8±1.75	1.87±0.0	2.29±0.06
MN_PD_RT (10)	226.22±51.08	13.57±3.06	1.94±0.02	2.26±0.03
Buffalo (20)	61.68±7.47	3.70±0.44	1.95±0.01	2.40±0.09

*and #indicates means between groups are statistically significant ($p<0.05$).

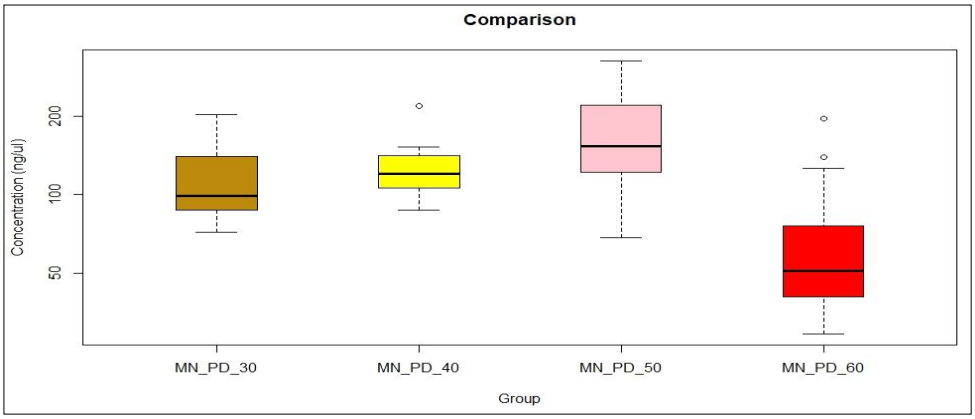
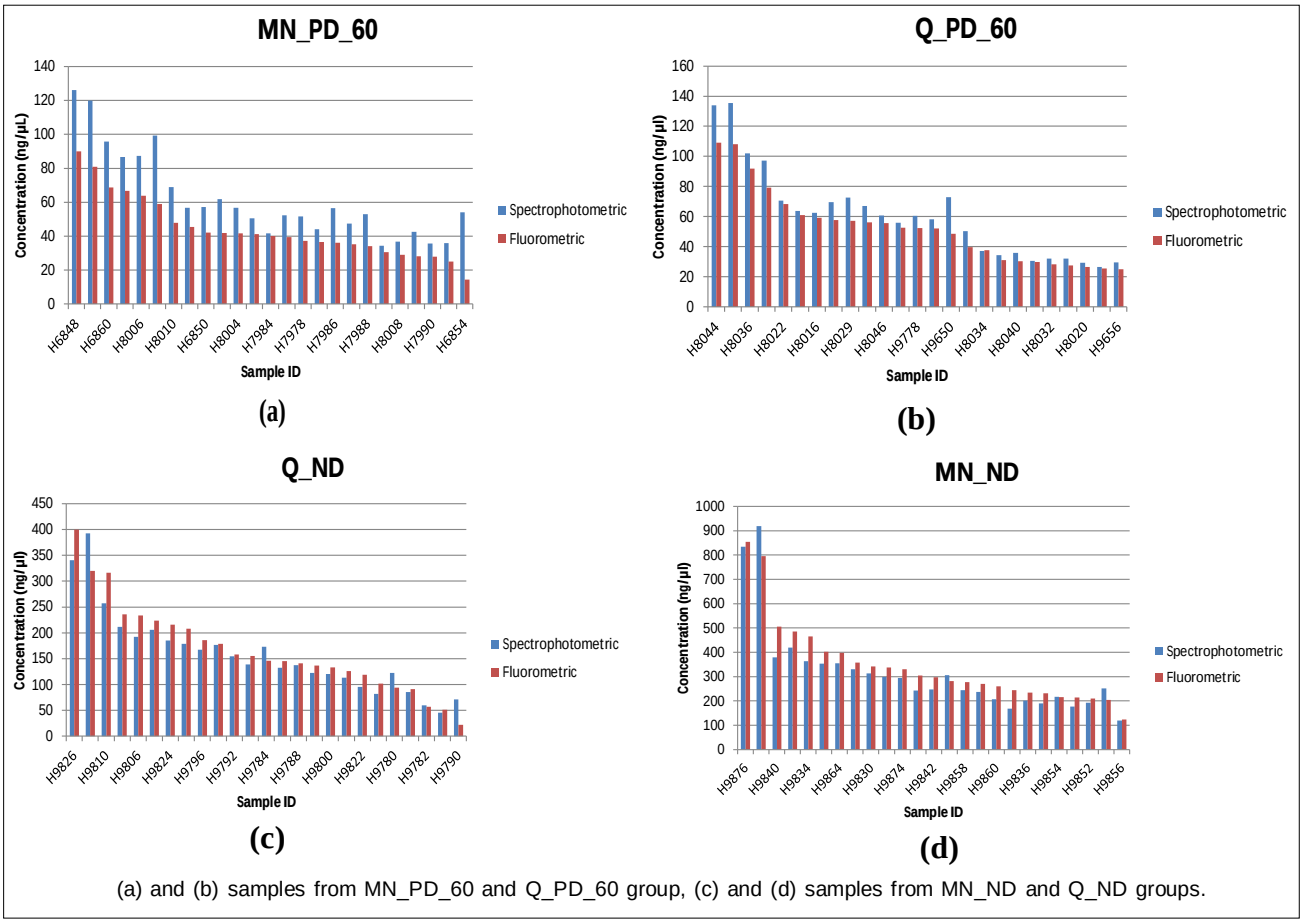
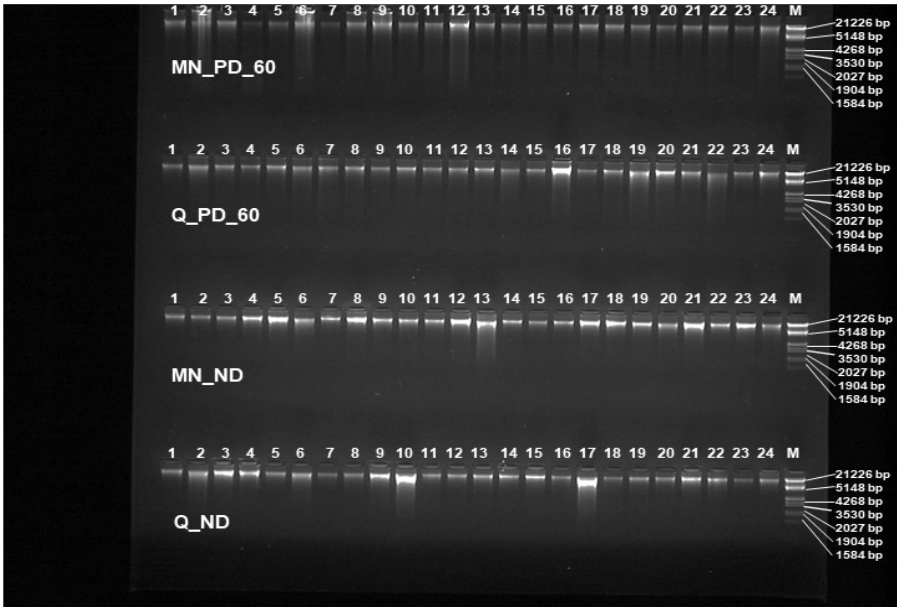


Fig 2: Effect of drying temperature on DNA concentration, extracted using NTK.



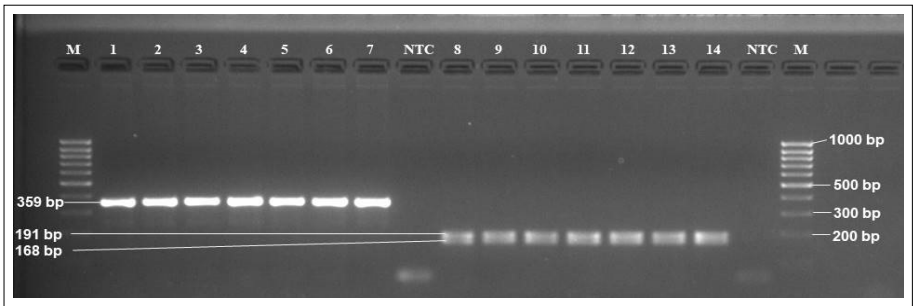
(a) and (b) samples from MN_PD_60 and Q_PD_60 group, (c) and (d) samples from MN_ND and Q_ND groups.

Fig 3: Comparison of Spectrophotometric and fluorometric measurements for samples from four groups.



(1-24: 24 samples from each group, M: Lambda DNA marker (EcoRI + HindIII digested, Thermo scientific).

Fig 4: An agarose gel image, showing DNA bands for samples belonging to four groups.



Lane 1-7 PCR amplified gDNA samples extracted using NTK, 8-14 RFLP with *TaqI*-two fragments of 191 bp and 168 bp, NTC- Negative control, M- 100 bp DNA ladder.

Fig 5: An agarose gel image, showing amplified DNA bands of 359 bp.

an overall reduction in the epidermis tissue available as a source of stored gDNA, making them difficult to process. Furthermore, the enzyme proteinase K and the lysis buffers are not harsh enough to disrupt the cartilage.

The specially designed preservative in the TSU protects the nucleic acids from degradation. Evaporation or leakage of this preservative fluid from TSUs may dehydrate the tissue sample. Usually, such drying is associated with water stress in the living systems. An investigation done by Wolkers *et al*. (1999) and Oldenhof *et al*. (2006) for the conformational changes that occurred in some of the cellular proteins due to water stress by Fourier Transform Infrared Spectroscopy (FTIR) revealed that when the process of drying is slow, such proteins get assembled into a higher ordered state and the sugar moieties form firm hydrogen bonds with each other to strengthen the network Wolkers *et al.* (1999); Oldenhof *et al.* (2006), which confer stability and protection to the viable macromolecules of the cell against water stress. Opposite to the above fact, when the drying is faster, the vice versa can be true, leading to poor gDNA yields. This may be the reason why good gDNA yield was obtained for already dried samples as the drying process for such samples was slow. Drying samples prior to DNA extraction is a well-known step for mushrooms Wand *et al.* (2017) and plant species Leonardo *et al.* (2016); Tai and Tanksley (1990), where it improves the overall quality and quantity of the extracted nucleic acids. Whereas, for animals, there are not sufficient evidences, whether drying can improve the same or not.

Examining the quality and quantity of the extracted DNA for all the groups, quality (260/280 nm ratio) did not g*et al*tered, however, the quantity was significantly higher for those samples which were not dried, showing drying of the samples with heat as a nonessential step before actual DNA extraction protocol.

Molecular dynamic simulation studies have shown the effect of temperature on DNA double helix (Driessen *et al*., 2014). The study revealed that, when an oligomer and a polymer of DNA incubated with a temperature range from 300 Kelvin (~27°C) to 400 Kelvin (127°C), the oligomer showed denaturation at the terminal ends, whereas the polymer was stable even at higher temperature (Kundu *et al*., 2012). However, the DNA polymer showed local denaturation bubbles throughout its entire length. This might be the reason that spectrophotometric DNA concentrations were higher compared to fluorometric measurements for MN_PD and Q_PD groups.

CONCLUSION

In the current study, ear tissue samples were dried before gDNA extraction, to get good DNA yield using two commercially available kits. Results showed that the tissue samples with *in vitro* incubation time around 221 to 250 days gave more gDNA yield. Additionally, the temperature driven fast drying is not essential prior to gDNA extraction, however drying temperature up to 50°C do not alter the yield much, suggesting easy transportation of TSUs up to 50°C. This is a preliminary study for drying animal ear tissue samples prior of gDNA extraction and it paves the way for further optimization with different drying methods.

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Conflict of interest: None.

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