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RESEARCH ARTICLE

Can DNA Barcode Study be Done from a Museum Specimen Fixed in a Formaldehyde Solution? A Case of *Emys orbicularis* (Linnaeus, 1758)

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Abstract: DNA barcoding, a molecular taxonomy technique, has been increasingly used by herptile taxonomists in recent years. In DNA barcoding studies with museum specimens, there are difficulties in achieving success in specimens that have been exposed to formaldehyde, which is usually used as a fixative, for a long time and intensively. Here we studied the effect of formaldehyde on the application of the DNA barcode method in *Emys orbicularis* specimens stored in 4% formaldehyde and 70% ethanol solution since 2008 and 2014. Sanger sequence analysis of tissues taken from samples stored in both ethanol and formaldehyde solution successfully yielded sequences of 623 bp. In conclusion, the use of ethanol solutions should be preferred for mid or long-term sample storage, especially in the context of molecular studies. In cases where the use of formaldehyde is unavoidable, it may be advisable to use extremely low concentrations to increase success in molecular research.

Anahtar kelimeler:

Sitokrom oksidaz I
Benekli kaplumbağa
DNA bakrodlama
Biyolojik koleksiyon
Türkiye

Formaldehit Çözeltisindeki Bir Müze Örneğinden DNA Barkod Çalışması Yapılabilir mi? *Emys orbicularis* (Linnaeus, 1758) Örneği

Öz: Bir moleküler taksonomi tekniği olan DNA barkodlama, son yıllarda herptil taksonomistleri tarafından giderek daha fazla kullanılmaktadır. Müze örnekleri ile yapılan DNA barkodlama çalışmalarında, genellikle fiksatif olarak kullanılan formaldehite uzun süre ve yoğun bir şekilde maruz kalan örneklerde başarı elde etmekte zorluklar yaşanmaktadır. Bu çalışmada, herptil koleksiyon materyali olarak 2008 ve 2014 yıllarından beri %4 formaldehit ve %70 etanol çözeltisinde saklanan Emys orbicularis örneklerinde formaldehitin DNA barkod yönteminin uygulanması üzerindeki etkisi araştırılmıştır. Hem etanol hem de formaldehit çözeltisinde saklanan örneklerden alınan dokuların Sanger dizi analizi sonucunda 623 baz çiftinden oluşan dizileri başarıyla elde edilmiştir. Sonuç olarak, özellikle moleküler çalışmalar bağlamında orta ve uzun süreli numune saklama için alkol solüsyonlarının kullanılmasının tercih edilmesi gerekmektedir. Formaldehit kullanımının kaçınılmaz olduğu durumlarda ise, moleküler araştırmalarda başarıyı artırmak için son derece düşük konsantrasyonların kullanılması tavsiye edilebilir.

Introduction

Since their inception, natural history collections have constituted an indispensable tool for taxonomists (Brooke, 2000), functioning as repositories for the entire spectrum of biological materials integral to taxonomic investigations and facilitating broad dissemination within the scientific community (Pulliandre *et al.*, 2012). Over a span of two and a half centuries, the most important mission of natural history museums and herbaria has been the preservation and dissemination of biological materials and data of scientific importance. However, the advent of the DNA revolution in the past two decades has engendered a novel challenge (Whitfield, 1999). Curators and scientists are currently confronted with two pivotal inquiries: (a) In instances where novel scientific methodologies necessitate access to the DNA of specimens, how may DNA extraction be conducted from specimens not initially preserved for genomic purposes? (b) When DNA sequences serve as a distinctive marker for specimen identification, how can the enduring association be sustained between the DNA barcode, which provides nomenclature to the user, and the specimens

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meticulously identified by taxonomists? (Pulliandre *et al.*, 2012).

The integration of morphological data with DNA barcode sequences constitutes a potent synergy for a broad spectrum of applications in biodiversity studies (Dayrat 2005, Will *et al.* 2005, Goldstein and DeSalle 2011, Miller *et al.* 2013, Riedel *et al.* 2013). The imperative determination of herptile species at the taxonomic level is indispensable for the comprehensive assessment of species assemblage, spatial distribution, and ecological vitality within a given ecological milieu. DNA barcoding, a molecular taxonomy technique, requires the use of short, predetermined gene sequences in combination with an established reference database (Hebert *et al.*, 2003). Although this method is known to show high efficiency and precision, Hawlitschek *et al.* (2016) emphasize that this method has some biases and limitations in its application. In recent years, numerous DNA barcoding studies on herptile taxa have been conducted (Chovanec and Grillitsch 1994; Beebee *et al.* 2005; Schlaepter *et al.* 2005; Smith *et al.* 2008; Vences *et al.* 2012; Murphy *et al.* 2013; Perl *et al.* 2014; Chambers *et al.* 2016; Hawlitschek *et al.* 2016; Zangl *et al.*, 2020; Ceríaco *et al.*, 2023). The merging of these molecular datasets significantly augments the repository of the global International Barcode of Life (iBOL) initiative, as posited by Ratnasingham and Hebert (2007).

In a review of the threat categories of turtles and tortoises according to the IUCN (International Union for Conservation of Nature), it was reported that 51.9% of the species had a threatened level Vulnerable or more (CR or EN) (Bayrakcı *et al.*, 2015; Rhodin *et al.*, 2018; IUCN, 2023). *Emys orbicularis* (Linnaeus, 1758) the European Pond Turtle is a Palearctic region-native freshwater turtle which has a wide distribution covers the Iberian Peninsula, the Maghreb, central Europe, southern France, Italy, the Baltic States, the Balkan peninsula, Türkiye and the Caspian Sea (Ficetola *et al.*, 2004; Fritz, *et al.*, 2009; Bayrakcı and Ayaz, 2014; Bayrakcı *et al.*, 2015; Escoriza *et al.*, 2020; Broggi, 2023). *Emys orbicularis,* inhabits temporary ponds and seasonal streams and, commonly found in permanent ponds and slow-flowing rivers which often surrounded by woodlands and marshes. According to the IUCN Red List *E. orbicularis* is listed as “Near Threatened” (Bayrakcı *et al.*, 2015; IUCN, 2023).

For various disciplines, including ecology, phylogenetics, biodiversity, evolutionary biology, and epidemiology, stored biological samples provide a valuable supply of genetic data. Formaldehyde solutions has been used to preserve samples, ranging from tissues to whole organisms. However, the sample's amplification of DNA and sequencing suffers by this preservation (Greer *et al.*, 1991). The development of new protocols for isolating DNA from formalin-fixed samples represents a promising avenue for increasing the ability to collect genetic information from such samples. In particular, the use of formalin-fixed samples curated in collections and museums is valuable to distinguish the impact of environmental changes on the genomic structure of biological populations (Totoiu *et al.*, 2020).

The ability of aqueous formaldehyde to inhibit the growth of parasitic microorganisms is another benefit (Fox *et al.*, 1985) yet using formalin-preserved samples for molecular testing is often challenging. Base deglycosylation is caused by the electrophilic formaldehyde's covalent alteration of DNA bases, which results in abasic regions that can break DNA strands (Lindahl and Andersson, 1972). PCR and DNA sequencing can also be inhibited by intrastrand and protein-DNA crosslinks created by formaldehyde (Dutta *et al.*, 2007).

Sequence artifacts, or apparent sequence changes that are distinct from the original sample can arise in DNA that has been effectively extracted from samples (Do and Dobrovic, 2015). Because it might be challenging to differentiate between real and artificial sequence changes, there is a higher chance of false-positive mutation calls (Wong *et al.*, 2013; Wong *et al.*, 2014)

In this study, we present the effect of different types of preserving solution to DNA barcoding studies and a local database for *Emys orbicularis* from the Çanakkale population.

Material and Methods

The samples of *Emys orbicularis* specimens were collected from 2 different localities and stored in 70% ethanol solution in the ÇOMU-ZLAR herptile collection (Table 1).

Table 1. Metadata of *Emys orbicularis* specimens of this study.

GenBank	Collection Code	Tissue Code	Species	Locality	Latitude	Longitude	Altitude (m)	Preserving Solution	DNA Concentration (µg/µl)	A260/230 (nm)	A260/280 (nm)
OR961469	2014/150	150	*Emys orbicularis*	Çanakkale	40.156867°	25.957941°	20	70% Ethanol	413.04	2.26	1.74
OR961478	2008/156	156	*Emys orbicularis*	Çanakkale	39.940815°	26.230843°	15	4% Formaldehyde	108.9	0.5	1.60

DNA Extraction

DNA isolations were carried out on muscular tissue sourced from the posterior extremities. It was performed according to the GeneMATRIX Tissue & Bacterial DNA Purification Kit's Sample preparation for formalin-fixed tissues procedure with minor modifications (Coombs *et al.*, 1999). In accordance with this preparation step, before the genomic DNA extraction, tissue samples were rinsed in phosphate-buffered saline (PBS) buffer to limit exposure to formalin gas and formalin pollution. The resulting DNA's quality and quantity were evaluated using the SPECTROstar Nano Spectrometer device. A straightforward yet efficient method of reducing sequence artifacts resulting from DNA lesions would be to employ particular DNA polymerases with low bypass efficiency over a range of DNA defects. GoTaq® DNA Polymerase, a proprietary Taq polymerase formulation that provides robust amplification comparable to and, in certain situations, better than that of traditional Taq, has been used in the current study.

PCR Amplifications

A 623-bp fragment from the 5′ region of the mitochondrial cytochrome c oxidase subunit I (COI) gene, recognized as the DNA barcoding region, was amplified using the degenerate universal barcoding primers dgLCO-1490 (5'-GGTCAACAAATCATAAAGAYATYGG-3') and dgHCO-2198 (5'-TAAACTTCAGGGTGACCAAARAAYCA-3') (Meyer, 2003). The PCR procedure commenced with an initial heating step at 94 ˚C for 2 min, followed by 35 cycles consisting of denaturation at 94 ˚C for 1 min, annealing at 54 ˚C for 45 s, and extension at 72 ˚C for 2 min. A final extension step at 72 ˚C for 10 min concluded the amplification process. Each 10 µL reaction mixture was composed of 0.05 ul of GoTaq® DNA Polymerase, 1,2 ul $MgCl_2$, 2 ul 5X Buffer, 0,8 ul dNTP, 0,5 ul of each primer (5 pmol/ml), and sterile water making up the final volume to 10 ul. The PCR products were then assessed for optimal fragment size through electrophoresis on a 2% agarose gel.

Sequencing

The purification of PCR products was carried out with the Thermo Fisher Scientific ExoSAP-IT PCR Product Cleanup Reagent following the supplier's protocol. Subsequently, unidirectional sequencing of these PCR products was conducted using the identical primers. The sequencing process occurred on a Thermo Fisher Scientific SeqStudio Genetic Analyzer, utilizing the Thermo Fisher Scientific BigDye Terminator v3.1 Cycle Sequencing Kit in accordance with the manufacturer's specifications.

Analyses

Analyses were performed based on 623 base pair fragments that were trimmed and quality checked. Nucleotide sequences were aligned and checked for read errors, insertions, and deletions. All analyses were performed using MEGA 11 (Tamura et al., 2021) to measure the proportion of correctly identified queries.

Results

A final sequence for cytochrome c oxidase I with a length of 623 base pairs was generated and deposited in the GenBank database under accession numbers OR961469 - OR961478 (Figure 1). No deletions or insertions were detected in the alignment of the sequences. Following translation of the protein-coding mitochondrial cytochrome c oxidase I sequences into protein; an analysis was performed for stop codons based on the vertebrate mitochondrial genetic code. No stop codons were detected, confirming that the cytochrome c oxidase I dataset encodes functional mitochondrial genes. Analysis of the nucleotide composition showed a G-C content of 40.50 percent for the cytochrome c oxidase I dataset.

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  1 gcattaagtc tactaatccg cgcagaactg agtcaaccag gagccctttt aggagatgac
 61 caagtctata atgttatcgt tacagcccat gccttcatta taatcttctt catggtcata
121 ccagttataa ttggtggatt tggaaattga cttgtaccat taataatcgg agcaccagat
181 atagcatttc cacgtataaa taatataagt ttctgacttt tacctccatc cctactacta
241 cttctagcat catcaggaat tgaagcaggg gcaggcacag gttgaactgt ataccccccg
301 ctagccggaa acttagctca tgccggtgcc tctgtagacc taactatttt ttctctccac
361 ttagctggtg tatcttcaat tttaggggct atcaatttta ttaccacagc aattaacata
421 aaatccccag ccatatcaca ataccaaaca cccctgtttg tatgatcagt acttattacc
481 gctgtcctat tactattatc attaccagta ctagctgcag gtatcactat actacttaca
541 gaccgaaact taaatacaac cttctttgac ccttcagggg gaggagaccc aatcctatat
601 caacacttat tctgattctt tgg
```

Figure 1. 623 base pair COI data of *Emys orbicularis* from formaldehyde and ethanol preserving solutions.

Discussion

As the exposure time to fixation increases, the number of direct and indirect reactions between DNA and the fixative also increases (Rogers *et al.*, 1990; Greer *et al.*, 1991; Forsthoefel *et al.*, 1992; Hamazaki *et al.*, 1993). Similarly, it has been demonstrated that the duration of fixation also influences the procedures to be carried out (Gavrilov and Razin, 2009). The results obtained from the current study showed that fixation of tissues with a low concentration of formaldehyde solution did not significantly reduce the amplification potential of DNA.

Blum (1894) is acknowledged for introducing formaldehyde as a tissue fixative. Currently, 10% neutral buffered formalin, a formulation of formaldehyde, is widely utilized as a universal fixative due to its effectiveness in preserving diverse tissue types and components. Nevertheless, endeavours to extract functional DNA from the tissues fixed with formalin have produced outcomes of varying success (Bramwell and Burns, 1988; Yagi *et al.*, 1996). In this case, one of the main factors contributing to the successful results of the specimen stored in formaldehyde solution is the use of about 3-4% formaldehyde, unlike the typical use of formaldehyde, which is usually around 10%.

Although we were successfully able to sequence from a formaldehyde specimen; for biological collections to fulfill their purpose, low concentrations of formaldehyde can be used, if necessary, after creating a "tissue inventory" by taking tissue from the specimens as a preliminary preparation for molecular studies before the specimens are fixed. In this way, we hope that since the samples can be stored intact, the need to obtain new samples from nature will be reduced and the collection samples can be used in future molecular studies.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contributions

Günay U.K.: Conceived, designed and performed analysis, wrote the paper. Pipilos A. and Keskin E.: Performed analysis and wrote the paper. Yakin B.Y. and Tok C.V.: Collected specimens and wrote the paper.

Ethics Approval

No alive specimens were used for this study. The specimens used in this study were found freshly dead during field studies in the past years (2008 & 2014) and have still been preserved in the ÇOMU-ZTAR herptile collection with 2008/156 & 2014/150 collection numbers.

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