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TECHNICAL NOTE

Trehalose improves PCR amplification of vertebrate nuclear DNA from historical allozymes

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Abstract Historical tissue collections represent potential resources for temporal genetic studies in evolutionary and conservation biology. Unfortunately, DNA from historical samples stored without modern genetic analyses in mind, such as frozen allozyme homogenates, are often degraded and contaminated with PCR inhibitors. Here, we report the successful use of trehalose for improving PCR amplification of degraded, vertebrate nuclear DNA extracted from cryopreserved allozymes. We amplified and sequenced two nuclear genes (*MLC2a* and *RPL12*) from allozymes of the Shasta salamander, *Hydromantes shastae*. Our results demonstrate the potential of trehalose as a tool for utilizing historical allozyme collections in modern genetic studies.

Keywords Conservation · Degraded DNA · PCR enhancer · Museum samples · Salamander

Museum collections provide exemplary resources for studies of phenotypic and genetic change through time, for understanding evolution on ecological timescales, and for evaluating species and populations of conservation concern (Suarez and Tsutsui 2004; Holmes et al. 2016). However, our ability to use museum collections for genetic studies is often limited by preservation methodology, particularly for samples collected before depositing tissue vouchers in ethanol became commonplace. DNA derived from historical tissues is often degraded, modified, or contaminated with PCR inhibitors. Still, studies have amplified DNA from traditionally preserved specimens including allozymes (Arbetman and Premoli 2011), formalin-fixed tissues (Hykin et al. 2015), bone (Wisely et al. 2004), feces (Snyder-Mackler et al. 2016), hair (Gilbert et al. 2004), feathers (Ellegren 1994), and arsenic-treated skins (Mundy et al. 1997). However, these methods have generally targeted plastid (mitochondrial or chloroplast) DNA, due to its relative abundance, or simple microsatellite repeats. Advances in PCR biochemistry that circumvent issues arising from DNA preservation will further expand the genetic information available in museum specimens to include nuclear sequence data.

Allozymes were the predominant markers for phylogenetic analysis until the 1990s (Schlötterer 2004), and, thus, substantial collections of historical allozymes exist. Recent work has demonstrated that chloroplast and nuclear microsatellite data can be generated from DNA extracted from cryopreserved plant allozymes (Arbetman and Premoli 2011), suggesting the potential to garner quality sequence data for other taxa and regions of the nuclear genome. Here, we demonstrate the efficacy of a trehalose additive, previously shown to improve cpDNA amplification in dried herbarium samples (Samarakoon et al. 2013), for improving PCR amplification of historical, low-quality, vertebrate nuclear DNA. As a PCR additive, trehalose increases thermal stability of *Taq* polymerase (Spiess et al. 2004), decreases the melting temperature of double-stranded DNA (Bezrukavnikov et al. 2014), and stabilizes single-stranded DNA backbones (Butler and Falke 1996; Bezrukavnikov et al. 2014).

We extracted whole genomic DNA using phenol-chloroform from 48 cryopreserved *Hydromantes shastae* allozymes primarily generated in 1978 (Wake et al. 1978). Some additional allozymes were generated from 1970 to 1990 (Wake

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DB, pers. comm.). Allozyme homogenates were stored frozen in either deionized water or buffer (0.1 M tris, 0.001 M EDTA, and 5×10^{-5} M NADP; pH 7.0). For comparison, we extracted high-quality, whole genomic DNA from eight frozen liver samples collected between 1990 and 2010 using a DNeasy Blood and Tissue Kit (Qiagen). For all samples, we attempted to use both standard and trehalose-treated reaction chemistry to PCR amplify two nuclear loci: myosin light chain 2 mRNA (*MLC2a;* 202 bp; C3F:5′-ATGCGTGTGAATTCCACATAATTG; C4R:5′-GAAGAACCCAACTGATGAATACCT) and 60S ribosomal protein L12 (*RPL12*; 350 bp; C31F:5′-ATTCCACTGCACCGCTATTGAT; C32R:5′-CCCAAGTTTGACCCTACAGAGAT; Fisher-Reid and Wiens 2011). We conducted standard 25 µL PCRs using 2% dimethyl sulfoxide, 0.4 µg/µL bovine serum albumin (BSA), 1X standard *Taq* reaction buffer (NEB), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM final concentrations for each primer, and 0.625U *Taq* polymerase (NEB). For reactions with trehalose, we used final concentrations of 1X TBT-PAR solution (150 mM trehalose, 0.2 µg/µL BSA, and 0.2% polysorbate-20; Samarakoon et al. 2013), 1X standard *Taq* reaction buffer, 1.5 mM $MgCl^2$, 0.2 mM dNTPs, 0.2 µM of each primer, and 0.625U *Taq* polymerase.

We ran PCRs at an initial temperature of 94 °C for 2 min, followed by 40 cycles of 94 °C for 30 s, primer specific temperature for 30 s (*MLC2a*: 51 °C; *RPL12*: 47 °C), and 72 °C for 30 s, followed by a final extension at 72 °C for 5 min. We purified successful amplicons using ExoSAP-IT (USB) and performed cycle sequencing reactions using Big Dye v3.1 sequencing chemistry (Applied Biosystems). We sequenced Sephadex™ G-50 cleaned products on an ABI Automated 3730xl DNA Analyzer (Applied Biosystems), examined electropherograms by eye, and assembled sequences in Sequencher v4.8. We deposited all successful sequences in GenBank (Accession Nos. MF470107-MF470113, MF470115-MF470126, MF470129-MF470147, MF470177, MF470180-MF470186). We compared standard and TBT-PAR reactions using chi-squared tests in R v3.3.3 (R Development Core Team 2017).

We successfully amplified both loci in all modern samples using both standard and TBT-PAR reactions. For allozymes, TBT-PAR improved amplification efficacy (Fig. 1). For *MLC2a*, we could not amplify allozyme samples under standard conditions, but we recovered 19 successful *H. shastae* sequences using TBT-PAR ($\chi^2=21.26$, $d.f.=1$, $P<0.001$; Table 1). For *RPL12*, TBT-PAR improved the amplification and sequencing success of allozyme samples from 6 to 11. This improvement was not statistically significant ($\chi^2=1.14$, $d.f.=1$, $P=0.28$), though it nevertheless almost doubled the success rate of PCR amplification. In both cases, some samples exhibited nonspecific binding or contamination by foreign DNA. DNA degradation in some long-held samples is likely too severe for successful Sanger sequencing. Furthermore, given the low-quality of DNA from allozyme samples, the risk of contamination by exogenous DNA is high. Still, despite these limitations, TBT-PAR substantially improves

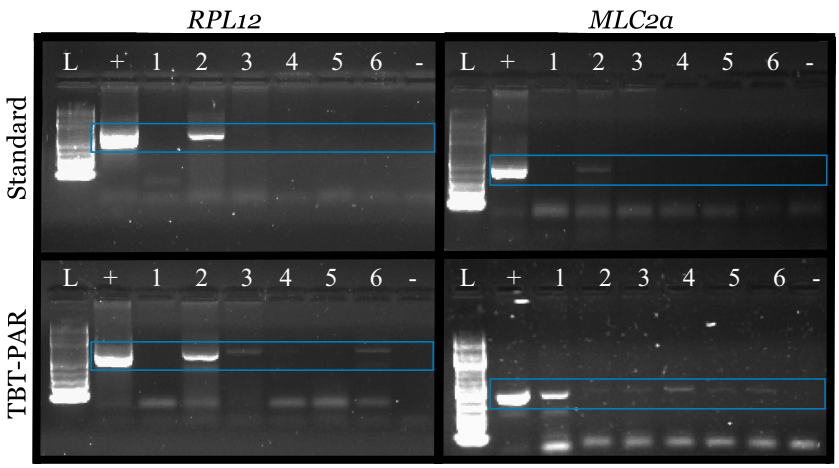

Fig. 1 Comparisons of standard and TBT-PAR supplemented PCR products visualized on 1.5% agarose gels. Each gel consists of a 100 bp ladder (NEB), PCR product froma modern sample (+), PCR products from historical allozymes (1–6), and a negative control (−). *Box* indicates desired amplicons

Table 1 Amplification success for *MLC2a* and *RPL12* using standard or TBT-PAR supplemented PCR

	Standard			TBT-PAR		
	None	Nonspecific	Successful	None	Nonspecific	Successful
MLC2a	47/48	1/48	0/48	28/48	1/48	19/48
RPL12	38/48	4/48	6/48	31/48	6/48	11/48

TBT-PAR improved the number of allozyme samples that amplified successfully

sequencing results from historical, low-quality DNA samples.

Our results demonstrate the value of trehalose in enhancing PCR amplification of nuclear DNA derived from vertebrate allozymes. The ability to sequence nuclear DNA from allozyme samples enables collections of cryopreserved homogenates to be utilized in temporal genetic studies and conservation genetic studies of rare, endangered, or difficult to sample species. Additionally, because TBT-PAR enhances DNA amplification from samples preserved by other techniques (e.g. dried herbarium samples; Samarakoon et al. 2013), it may prove broadly useful for improving PCR chemistry across a range of taxa and preservation methods.

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