

TECHNICAL NOTE

Co-amplification of cytochrome *b* and D-loop mtDNA fragments for the identification of degraded DNA samples

DONGYA Y. YANG and CAMILLA F. SPELLER

*Ancient DNA Laboratory, Department of Archaeology, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia, Canada V5A 1S6***Abstract**

We propose a simple and effective approach to simultaneously co-amplify both cytochrome *b* and D-loop fragments to evaluate DNA preservation and to monitor possible contaminations in the analysis of degraded animal DNA samples. We have applied this approach to over 200 ancient salmon samples and 25 ancient whale DNA samples, clearly demonstrating its multiple benefits for analysis of degraded DNA samples, and the ease in which co-amplification can be optimized for different taxa. This simple, cost-efficient and genomic DNA-saving approach can be used routinely in the analysis of minute and degraded DNA samples in wildlife forensics, food inspection, conservation biology and ancient faunal remains.

Keywords: animal mtDNA, cytochrome *b*, D-loop, PCR amplification, species identification

Received 14 November 2005; revision received 4 January 2006; accepted 23 February 2006

Although polymerase chain reaction (PCR)-based DNA techniques are widely used for animal species and subspecies identifications (Palumbi & Cipriano 1998; Bellis *et al.* 2003), difficulties are still encountered when analysing minute and degraded DNA samples such as hair and fecal samples from wildlife forensic investigations or DNA from ancient remains (Teletchea *et al.* 2005). DNA degradation and contamination with extraneous DNA are the two most overwhelming challenges; they can easily lead to false results (Cooper & Poinar 2000). Consequently, the authentication of degraded DNA results always requires a heavy burden of proof (Paabo *et al.* 2004). Quantification of original DNA templates and duplication of PCR amplifications are considered to be two important steps in ensuring the reliability of ancient DNA results. However, both of these processes can be costly and time-consuming.

In this study, we propose a simple and effective approach to evaluate DNA degradation and reliability of DNA identification by simultaneously co-amplifying two fragments of mitochondrial DNA (mtDNA) from the cytochrome *b* gene (*cyt b*) and control region (D-loop), in a single PCR. The key to successful co-amplification is the design of four optimal primers to target group-specific fragments of less

than 200–300 bp. Primer design computer programs (including those free online such as OLIGO 3 or NETPRIMER) can be used to facilitate the primer design process, based on readily retrieved reference DNA sequences from GenBank and other online databases. Ideally, the *cyt b* fragment should be shorter than the D-loop fragment, making the conservative *cyt b* fragment also serve as an internal control for PCR amplification. Balanced co-amplification of the two fragments can be realized by simply adjusting the primer concentration ratios for the longer and shorter DNA fragments, and unevenly amplified samples within the same sample set can be indicative of differential DNA preservation. This new approach can quickly visualize the extent of DNA degradation using an electrophoresis gel and identify possible sample contamination through subsequent sequencings of both fragments.

To test the applicability and the feasibility of this technique, we applied it to ancient salmon and whale DNA from archaeological sites in British Columbia, Canada. These particular samples were considered appropriate because (i) most of these samples have been previously subjected to multiple simplex PCR amplifications; (ii) these ancient samples represent some examples of extremely degraded DNA; (iii) they represent two very different groups of species — closely related species of salmon (within the same genus) and distantly related species of

Correspondence: Dongya Yang, Fax: (604) 291-5666; E-mail: donyang@sfu.ca

	Primer	Sequence (5'–3')	bp	Amplicon
Whale	F1 (F)	ATGACCAACATCCGAAAAACAC	22	F1 + R182
cyt <i>b</i>	R182 (R)	GTTGTTGTGTCTGGTGTGTAGTGTATT	27	(182 bp)
Whale	F22 (F)	CCACCATCAGCACCCAAAGC	20	F22 + R258
D-loop	R258 (R)	TGCTCGTGGTGTARATAAATTGAATG	25	(237 bp)
Salmon	cyt <i>b</i> 5 (F)	AAAATCGCTAATGACGCACTAGTCGA	26	cyt <i>b</i> 5 + cyt <i>b</i> 6
cyt <i>b</i>	cyt <i>b</i> 6 (R)	GCAGACAGAGGAAAAAGCTGTTGA	24	(168 bp)
Salmon	Smc7 (F)	AACCCCTAAACCAGGAAGTCTCAA	24	Smc7 + Smc8
D-loop	Smc8 (R)	CGTCTTAACAGCTTCAGTGTATGCT	26	(249 bp)

Table 1 Primers for co-amplification for whale and salmon species

F is for the forward primer and R is for the reverse primer. The salmon primers are from Yang *et al.* (2004).

whale (in different families). The salmon samples are dated to 800–7000 bp from Namu and Keetley Creek sites (Yang *et al.* 2004; Speller *et al.* 2005) and the whale samples are dated to 200–3500 bp from Barkley Sound of Vancouver Island (Monks *et al.* 2001).

We followed vigorous contamination control measures for bone preparation and DNA extraction. A single vertebra from the salmon samples (0.3–0.6 g) and approximately 1 g of whale bone were decontaminated using the previously published methods (Yang *et al.* 2004). Each bone sample was ground into fine powder before it was incubated overnight at 50 °C with 2–5 mL proteinase K digestion buffer (0.5 M EDTA pH 8.0, 0.5% SDS and 0.5 mg/mL proteinase K) in 15-mL tube. We used a modified silica-spin column method for DNA extraction (Yang *et al.* 1998).

We used the online program PRIMER 3 (Rozen & Skaletsky 2000) to design the primers and NETPRIMER (PREMIER Biosoft International) to exclude the possibility of forming primer-dimers. To our surprise, we found more than the half of the primers that were previously used for simplex PCR could be used without modification for co-amplification (Table 1). PCR amplifications were conducted in an Eppendorf Mastercycler in a 25- μ L or 50- μ L reaction volume containing 50 mM KCl and 10 mM Tris-HCl, 2.5 mM MgCl₂, 0.2 mM dNTP, 1.5 mg/mL BSA, 2.5 or 5 μ L of DNA sample and 1.25 or 2.5 U AmpliTaq Gold (Applied Biosystems), respectively. To optimize PCR, we fixed the primer concentration of the longer fragment at 0.3 μ M for all amplifications, while the primer concentration of the shorter fragment ranged from 0.3 μ M to 0.1 μ M to achieve even co-amplification. The ratio of the primer sets varied from 1:1, 3:2 and 3:1, to even greater ratios when the concentration of the longer fragment was increased to greater than 0.4 μ M.

PCR was run at 40–60 cycles with cycle conditions of 94 °C for 30 s, 55 °C for 30 s and a 72 °C extension for 40 s, with an initial denaturing at 95 °C for 12 min. Five microlitres of PCR product was separated by electrophoresis on a 2% agarose gel to evaluate the result of the co-amplification. Using different ratios of the two primer sets and optimized

PCR conditions, even amplification of the two fragments was achieved for the majority of the samples in this study. The primer ratio seemed to be most affected by the size difference between the two different fragments: higher primer ratios were needed to evenly amplify two DNA fragments with a large size difference (Bataille *et al.* 1999). The co-amplifications of some of the salmon DNA samples are displayed in Fig. 1.

PCR products were purified using QIAquick or MinElute PCR Purification kit (QIAGEN), and both the cyt *b* and D-loop fragments were directly sequenced from the co-amplified PCR products (using either forward, reverse or both primers). Clear sequencing signals were generally obtained although sequencing electropherograms showed mixed beginning in some samples, possibly due to the increased chance of primer-dimer formation during co-amplification. However, this problem was readily overcome by designing a new sequencing primer. Thus, the presence of the two DNA fragments within the PCR products did not seem to interfere with direct sequencing of the target DNA fragment when using a product-specific primer.

In over 200 salmon samples and 25 whale sample, the co-amplification sequences were consistent with those from simplex PCR sequences excepts for one whale sample (see below) (for the salmon samples, see Yang *et al.* 2004). In total, five salmon species [sockeye (*Onchorynchus nerka*), coho (*Onchorynchus kisutch*), chum (*Onchorynchus keta*), pink (*Onchorynchus gorbuscha*) and chinook (*Onchorynchus tshawytscha*)] and four whale species [humpback whale (*Megaptera novaeangliae*), grey whale (*Eschrichtius robustus*), blue whale (*Balaenoptera musculus*) and killer whale (*Orcinus orca*)] were identified from the ancient remains.

The technique of co-amplification has several key benefits when applied routinely to degraded DNA samples:

- 1 One obvious advantage is that the co-amplification technique provides valuable information about DNA preservation. Fig. 1 shows uneven co-amplification of some samples, which is most likely due to differential



Fig. 1 Negative image of a SYBR-Green stained, 2% agarose gel of co-amplified PCR products. Samples 1–19 represent DNA samples extracted from ancient salmon remains, BK is the blank extract and N is the PCR negative control. Sample 19 displays amplification failure of both bands, while sample 13 displays amplification failure of the longer D-loop fragment. The markers on the left are 100-bp ladders (from Invitrogen).

DNA degradation and preservation. For degraded DNA samples, there is a reverse relationship between the length of templates and the amount of templates: the longer the fragment, the fewer are preserved intact (Hoss *et al.* 1996). If the DNA is relatively more heavily degraded, the longer DNA fragment will be less favourably amplified, resulting in weaker amplifications, while those samples with a better DNA preservation would show stronger amplification of the longer fragments (Fig. 1, samples 17 and 18). The latter is further confirmed when the same PCR condition is applied to modern salmon DNA samples (data not shown). When the opposite pattern is consistently visible in some degraded samples after the PCR condition is optimized and fixed, it may indicate that DNA in these samples is better preserved (Fig. 1, samples 17 and 18).

- 2 The co-amplification also makes the detection of sample contamination much more obvious if both DNA fragments indicate two different species or subspecies. Among all of the analysed samples, we detected contamination in only one whale sample where the amplified *cyt b* fragments turned out to be unexpectedly from pig DNA. This contamination was readily detected in the discrepancy of species IDs when the D-loop fragment was sequenced. We expect the co-amplification approach to effectively detect contamination by PCR products if it should occur.
- 3 Moreover, the co-amplification technique confirms the species identity using two different fragments from the same PCR tube. For salmon sample 6 (Fig. 1), an insertion in the D-loop sequence caused some difficulty in species

identification, although the sequence was most similar to pink salmon; this species ID was readily confirmed by a pink salmon *cyt b* sequence match. In a subsequent D-loop simplex amplification, sample 13 was also found to contain this insertion, which may in part account for the amplification failure of the D-loop fragment during the co-amplification process.

- 4 The success rate of the co-amplification may also indicate the species diversity of DNA samples. As expected in this study, the success rate of co-amplification was generally higher (over 90%) for closely related salmon samples than for the more distantly related whale samples (70%). It is expected that any given D-loop primer set will only work efficiently with certain closely related species and may bind less effectively with other species.

In conclusion, given that the new co-amplification approach is straightforward to design and apply, with multiple advantages in estimating DNA degradation and detecting sample contamination, we strongly propose a routine use of this new approach to increase the reliability of DNA identification from degraded animal DNA samples.

Acknowledgements

Our special thanks go to Aubrey Cannon, Alan McMillan and Brian Hayclen for making archaeological salmon and whale bones available for the study. We thank Kathy Watt, Sarah Dersch, Sarah Padilla and Kelly Kim for technical assistance. This research was supported in part by research grants (DY Yang) including Social Science and Humanities Research Council of Canada's RDI fund, and SFU Discovery Park Fund.

References

- Bataille M, Crainic K, Leterreux M, Durigon M, de Mazancourt P (1999) Multiplex amplification of mitochondrial DNA for human and species identification in forensic evaluation. *Forensic Science International*, **99**, 165–170.
- Bellis C, Ashton KJ, Freney L, Blair B, Griffiths LR (2003) A molecular genetic approach for forensic animal species identification. *Forensic Science International*, **134**, 99–108.
- Cooper A, Poinar HN (2000) Ancient DNA: do it right or not at all. *Science*, **289**, 1139.
- Gilbert MTP, Bandelt H-J, Hofreiter M, Barnes I (2005) Assessing ancient DNA studies. *Trends in Ecology & Evolution*, **20**, 541–544.
- Monks GG, McMillan AD, Claire DES (2001) Nuu-chah-nulth whaling: archaeological insights into antiquity, species preferences, and cultural importance. *Arctic Anthropology*, **38**, 60–81.
- Paabo S, Poinar H, Serre D *et al.* (2004) Genetic analyses from ancient DNA. *Annual Review of Genetics*, **38**, 645–679.
- Palumbi SR, Cipriano F (1998) Species identification using genetic tools: the value of nuclear and mitochondrial gene sequences in whale conservation. *Journal of Heredity*, **89**, 459–464.
- Rozen S, Skaletsky HJ (2000) PRIMER 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, NJ.
- Speller CF, Yang DY, Hayden B (2005) Ancient DNA investigation of prehistoric salmon resource utilization at Keatley creek, British Columbia, Canada. *Journal of Archaeological Science*, **32**, 1378–1389.
- Teletchea F, Maudet C, Hanni C (2005) Food and forensic molecular identification: update and challenges. *Trends in Biotechnology*, **23**, 359–366.
- Yang DY, Eng B, Wayne JS, Dудар JC, Saunders SR (1998) Improved DNA extraction from ancient bones using silica-based spin columns. *American Journal of Physical Anthropology*, **105**, 539–543.
- Yang DY, Cannon A, Saunders SR (2004) DNA species identification of archaeological salmon bone from the Pacific Northwest Coast of North America. *Journal of Archaeological Science*, **31**, 619–631.