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Short Communication

Contamination and chimerism are perpetuating the legend of the snake-eating cow with twisted horns (*Pseudonovibos spiralis*). A case study of the pitfalls of ancient DNA

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In 1994, several unusual horns collected from markets in Vietnam and Cambodia were described as a new genus and species of wild bovid, *Pseudonovibos spiralis* (Peter and Feiler, 1994a,b). The horn sheaths possessed a unique and distinct lyriform twist, as well as annulations throughout their entire length. This animal, named "linh duong" in Vietnam or "kting voar" in Cambodia (but see Brandt et al., 2001), was known only from detached horn sheaths or frontlets (partial frontal bones with bony horn cores) with horn sheaths. This lack of anatomical information was largely responsible for the confusion regarding the taxonomic status of this species, with morphologists debating whether it was a close relative of the tribe Antilopini (gazelles) (Peter and Feiler, 1994a), Caprini *sensu lato* (goats, sheep, and allies) (Nadler, 1997), or Bovini (oxen, bison, and buffaloes) (Dioli, 1997; Timm and Brandt, 2001). Given both the paucity and variable taxonomic interpretation of the morphological data, hopes were initially high that DNA sequence would resolve the issue of *Pseudonovibos*' taxonomic status and phylogenetic position. However, three ensuing DNA sequencing studies using different putative *P. spiralis* specimens gave rise to mutually incompatible hypotheses:

(1) Hammer et al. (1999), using a 415-bp DNA fragment of the mitochondrial cytochrome *b* gene, proposed affinities with the Caprini *sensu lato*. However, Hassanin and Douzery (2000) challenged the authenticity of the Hammer et al. (1999) sequence and interpreted it as the result of DNA contamination from chamois (*Rupicapra*) in the laboratory.

(2) Hassanin et al. (2001) revealed that some horns assigned to *P. spiralis* are simply cow horns that had been artificially carved and twisted. Two DNA markers were sequenced from four trophies of *P. spiralis* collected in Indochina during 1925: a 243-bp fragment of the mitochondrial cytochrome *b* and a 327-bp fragment of the nuclear lactoferrin gene. The phylogenetic results showed that the enigmatic horns of the linh duong belonged to domestic cattle (*Bos taurus*) (Hassanin et al., 2001). Morphological inspection indicated that horn sheaths, originally smooth, were carved to create the annulations, while the twist in the upper part of the horns was made by artificial torsion (Thomas et al., 2001). This raised the question of whether all horns of the linh duong are fraudulent or not. In other words, did the species *P. spiralis* ever really exist (see review in Brandt et al., 2001)?

(3) Most recently, Kuznetsov et al. (2001) suggested that *P. spiralis* was a new species of buffalo based on a 962-bp fragment of the mitochondrial 12S rRNA gene. However, one of us (Hassanin, 2002) demonstrated that the putative sequence of *P. spiralis* was a chimera obtained from three different species: *B. taurus*, *Bubalus bubalus* (domesticated Asian water buffalo), and *Saiga tatarica* (saiga antelope). In addition, several factors indicated that their specimen was artificially made using horns and a frontlet from domestic cattle, *B. taurus*.

The ongoing and increasingly heated controversy surrounding *P. spiralis* has been covered in Science (Malakoff, 2001), Nature (Whitfield, 2002), and the New York Times (Mydans, 2002), with emphasis on both the folkloric portrayals of a snake-eating cow-like creature

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some have linked to *P. spiralis* as well as its taxonomic status. In a recent paper published in this journal, Kuznetsov et al. (2002) suggested that *P. spiralis* was the sister taxon of Bubalina, a subtribe which includes the buffaloes of Asia (genus *Bubalus*) and Africa (genus *Syncerus*). They proposed to include this species in a newly erected subtribe, Pseudonovibovina. However, the 12S rDNA sequence of *P. spiralis* used in this paper (GenBank Accession No. AF231029) was the same as that reported by Kuznetsov et al. in *Naturwissenschaften* (2001), which has been shown to be chimeric (see above) and, in addition, impossible to generate as continuous, organismal sequence using the strategy outlined by these authors (see below). Although this has been noted previously (Hassanin, 2002), the continued use of the putative *P. spiralis* 12S rDNA sequence in peer-reviewed journals and the concomitant implication of its authenticity warrant reiteration of its dubious origin.

Fig. 1A provides a schematic representation of the amplification strategy employed by Kuznetsov et al. (2001). These authors purportedly amplified and sequenced four overlapping sections (A–D) of the 12S rRNA gene from a *P. spiralis* specimen, concatenating them using the regions of overlap for homology inference. As shown by Hassanin (2002) and again in Fig. 1, these four fragments do not overlap when the primers reported by Kuznetsov et al. (2001) are excised from the terminal regions of the four resulting sequences. This is true whether the primers are mapped onto *B. taurus* 12S or the *P. spiralis* sequence reported by Kuznetsov et al. (2001). In other words, even if the organismal sequence *exactly* matched the sequence as reported by these authors, it is impossible to generate it in its entirety using their procedure. This is because, as shown in Fig. 1B, overlap between contiguous fragments can only occur if terminal sequences representing unexcised oligonucleotide sequences are considered. Given that the primers employed are exact reverse complements of each other where they overlap, identical overlap is predetermined but entirely spurious. In this situation, adjacent sequences from unrelated taxa would appear to overlap perfectly, regardless of how many positions were non-identical between these taxa at the primer binding sites, resulting in chimeric sequence. Of the 962 bases reported as continuous *P. spiralis* DNA, 45 of these represent unexcised oligonucleotides. We emphasize that these 45 bases, 31 of which comprise the only "overlap" between any two adjacent PCR products, *do not represent original* (*i.e., organismal*) *template DNA*. The inclusion of these bases in the sequence reported by Kuznetsov et al.

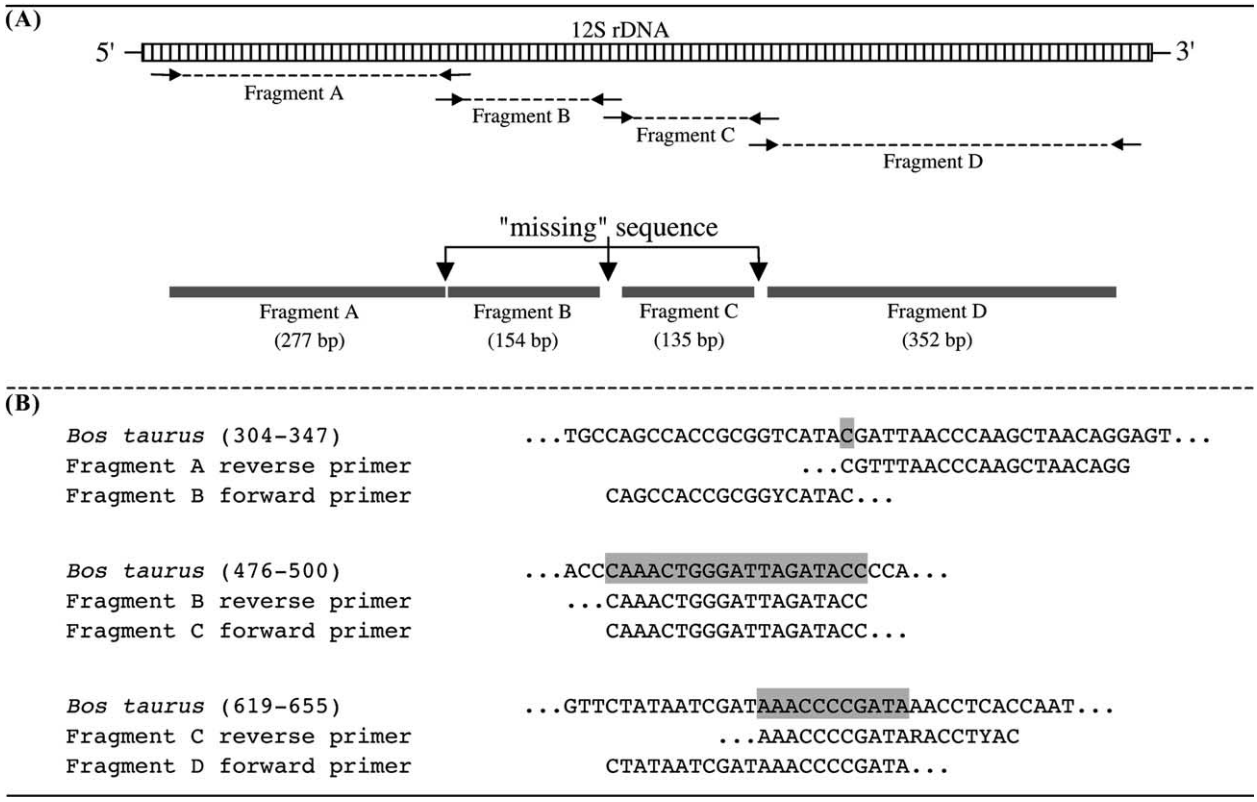

Fig. 1. Amplification strategy of Kuznetsov et al. (2001). (A) Schematic overview. (B) Positions of six of the eight primers against the 12S rRNA gene in *Bos taurus*. Numbers in parentheses represent the location of each sequence shown in the mitochondrial genome of *Bos taurus* (Anderson et al., 1982). Original template (organismal) sequence highlighted in gray would not be sequenced if each adjacent fragment (A–D; see Fig. 1) were amplified and sequenced individually, and therefore no overlap would be attained. An additional 14 bases of unexcised oligonucleotide sequence were included at the 5′ end of the *Pseudonovibos* sequence reported by Kuznetsov et al. (2001).

(2001) and their use in concatenating the four independently amplified and sequenced PCR products is therefore invalid.

Given the absence of overlapping organismal sequence between any two adjacent fragments, the hypothesis that each is derived from the same template sequence and is not the result of exogenous contamination can and should be tested by (1) checking each individual fragment (trimmed of oligonucleotide sequences) against all published sequences (e.g., performing a BLAST search; Altschul et al., 1997) and (2) performing separate phylogenetic analyses on each individually amplified fragment. Hassanin (2002) carried out such a test on the *P. spiralis* sequence reported by Kuznetsov et al. (2001), concluding that it is composed of contaminant DNA from *Bubalus bubalus* (fragments B and C) and *Saiga tatarica* (fragment D), with fragment A representing DNA identical to that of *Bos taurus* and possibly derived from the actual specimen sampled (additional evidence in support of this can be found in Hassanin, 2002). Thus, as with previous reports of novel *P. spiralis* DNA sequence, this appears to be a simple case of contamination, with no molecular evidence that the specimen sampled represents anything other than a domestic cow (*B. taurus*).

This example also illustrates another facet of the issue of overlap in ancient/degraded DNA studies. As we have shown, no overlap between linearly adjacent DNA fragments could have been obtained using the primers and amplification strategy of Kuznetsov et al. (2001). However, we suggest that even if the terminal regions representing unexcised oligonucleotides *did* represent organismal sequence, overlap is still insufficient for the confident assembly of concatenated DNA, particularly with respect to the aims and context of the original study. As shown in Fig. 1B, ''overlap'' (as mistakenly inferred in the original study) between any two adjacent fragments ranges from one (A and B) to 19 (B and C) bases. We are unaware of any published standards regarding the issue of sufficient overlap in any DNA study (let alone ancient DNA studies) that involve concatenation. Most investigators would likely agree that a one-nucleotide overlap is questionable, at best, and should be regarded with extreme caution. Beyond that, the question has no clear answer. Ideally, overlapping regions would span at least one diagnostic or unique mutation. However, novel mutations within short stretches of DNA in relatively slowly evolving markers such as 12S are not to be expected among closely related taxa (e.g., the two species of *Bubalus* differ at only 7 out of 937 positions), making contamination all the more difficult to identify. The ''overlapping'' regions reported by Kuznetsov et al. (2001) are invariant at all three junctures (A/B, B/C, and C/D) in all of the taxa included in their study, with one exception (a single nucleotide difference in *Tetracerus* at C/D). In this situation, particularly in ancient DNA studies, it is incumbent on researchers to acknowledge and test the possibility of chimerism (in addition to conducting standard negative controls), which in the case of the *P. spiralis* 12S, is readily evident (Hassanin, 2002).

The evidence against the authenticity of the *P. spiralis* 12S rDNA reported by Kuznetsov et al. (2001) is overwhelming. We propose that it be confirmed using new primers specifically designed to encompass (and extend well beyond) the regions of putative overlap between fragments A and B and C/D. Furthermore, in accordance with established protocols for determining ancient DNA sequences (e.g., Cooper and Poinar, 2000), separate extractions, amplifications, and sequencing should also be conducted in an independent lab dedicated to ancient DNA extraction and amplification to confirm reproducibility. Until the 12S sequence reported by Kuznetsov et al. (2001, 2002) can be validated, their phylogenetic conclusions and the taxonomic status of *P. spiralis* should be regarded with continued incredulity.

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