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Sorting out tigers (*Panthera tigris*): mitochondrial sequences, nuclear inserts, systematics, and conservation genetics

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Abstract

Sequences from complete mitochondrial cytochrome *b* genes of 34 tigers support the hypothesis that Sumatran tigers are diagnostically distinct from mainland populations. None of the latter, including Bengals, Siberians, or Indochinese tigers, were found to have fixed diagnostic characters. Phylogenetic analysis of these sequences confirms these results. Within the framework of a phylogenetic species concept, current evidence thus supports the recognition of two distinct taxa, and within the context of this definition they could be ranked at the species level. This paper also documents a previously unrecognized nuclear insert of mitochondrial DNA that includes, minimally, mitochondrial homologues of a control region that lacks the feline mitochondrial repeat sequences, a complete cytochrome *b* gene, and complete $tRNA^{Thr}$ and $tRNA^{Pro}$ genes. In a phylogenetic analysis of the nuclear cytochrome *b*-like sequences and various feline mitochondrial sequences, the nuclear insert clusters with lion mitochondrial cytochrome *b* sequences, which suggests the insert is at least as old as the split between lions and tigers. The results of this study emphasize the importance of doing more to conserve Sumatran tigers. Because they are underrepresented in zoos relative to Bengals and Siberians, an effort should be made to increase captive breeding stocks of Sumatrans. That Sumatrans are a distinct taxonomic entity relative to mainland populations can be used in educational programs to increase conservation efforts within Indonesia.

INTRODUCTION

Tigers (*Panthera tigris*) are among the most well known of the 'megacharismatic' vertebrates. They are also internationally acknowledged as being under severe threat of extinction in the wild (Groombridge, 1993) due to extensive habitat loss (Seidensticker, 1986; Tilson & Seal, 1987) and harvesting for the Asian medicinal market (McMahan, 1986; Houji & Helin, 1986; Kenney *et al.*, 1995; Thapar, 1997; Tilson, Traylor-Holzer & Jiang, 1997). Tigers are, therefore, the target of a global conservation effort.

Five extant subspecies of tigers are currently recognized: the South China (*P. t. amoyensis*), Siberian (*P. t. altaica*), Bengal (*P. t. tigris*), Indochinese (*P. t. corbetti*), and Sumatran (*P. t. sumatrae*). Several other subspecies – those on Java, Bali, and in central Asia – are already extinct (Seidensticker, 1986; Joslin, 1986). Historically, variation in pelage color and body size has formed the basis for subspecies boundaries (Mazak, 1981; Seidensticker, 1986; Herrington, 1987), but there is still no comprehensive, modern analysis of geographic variation in tigers. The variation that has been studied, including morphometric analysis of skull measurements (Herrington, 1987), is questionably diagnostic for the different forms and some of it may be clinal (Hemmer, 1987). Likewise, available molecular evidence suggests that extant tigers are extremely similar genetically, but the techniques used to date have been unable to establish whether any of the currently recognized subspecies are genetically distinct from one another (O'Brien *et al.*, 1987; Goebel & Whitmore, 1987; Wayne *et al.*, 1989). Thus, the taxonomic status of these geographic subspecies remains uncertain.

Understanding the diagnostic limits to geographic patterns of variation is a critical concern for conservation biology because of its essential role in delimiting discrete taxonomic units in nature (Ryder, 1986; Cracraft, 1989, 1997; Geist, 1992; Vogler & DeSalle, 1994; Barrowclough & Flesness, 1996). *In situ* and *ex situ* management plans of tigers require this information (Foose, 1987; Maguire & Lacy, 1990). Decisions about allocating space for captive breeding in zoos (Foose & Seal, 1986; Maguire & Lacy, 1990), for example, or breeding programs for maximizing genetic diversity, depend on knowing whether the different forms of tigers

are diagnosably distinct. Regulating the illegal trade of tigers and identifying the geographic sources of that trade also depend on knowledge of diagnosable taxa and the genetic or morphological markers that identify them. Finally, countries have a vested interest in identifying the diagnosable taxa present within their borders for a number of reasons: discerning patterns of diversity and endemism, formulating conservation management plans, as well as establishing a basis for environmental education programs (Cracraft, 1997).

We undertook this study to investigate whether any of the currently recognized subspecies of tigers are diagnosably distinct when using DNA sequencing techniques with sufficient power to resolve phylogenetic patterns in closely related organisms. An affirmative answer to that question then leads one to investigate the issue of the interrelationships of these taxa, their historical biogeography, and the tempo of branching events that may underlie current patterns of taxonomic and genetic distinctness.

During the course of this study we discovered an heretofore unrecognized fragment of tiger mitochondrial-like sequences, containing a mitochondrial-like control region (D-loop) lacking the feline repeat sequences, a complete cytochrome *b* gene, and complete $tRNA^{Thr}$ and $tRNA^{Pro}$ genes. For reasons to be discussed, we infer these to have been inserted into the nuclear genome. Because the accuracy of any statement about patterns of genetic variation, and their relevance for conservation genetics, depends on distinguishing between mitochondrial and nuclear sequences, we characterize these mitochondrial-like sequences and then compare them to their mitochondrial counterparts.

MATERIALS AND METHODS

Samples and provenance

Blood samples of a lion (*Panthera leo*) and 34 captive tigers (except for *P. t. amoyensis* which was not available to us), all of presumed known parentage, were provided by the following sources [information in parentheses after the laboratory code for individual specimens includes: sex, zoo or source, and an identifying number such as local zoo number (ID) and studbook number (SB), when available]:

Panthera tigris tigris: B2 (F, Topeka Zoo ID 1182 via Brookfield Zoo ID 8-G-3,4; SB 8411), B3 (F, Atlanta Zoo ID 750275 via Brookfield Zoo ID13-I-4), B4 (M, Atlanta Zoo ID 750175 via Brookfield Zoo ID 13-J-4), B5 (F, Cincinnati Zoo, ID 189158, SB 360), B6 (M, wild caught Nagarhole Park, India, from S. J. O'Brien, ID Pti-102), B7 (F, wild caught Nagarhole Park, India, from S. J. O'Brien, ID Pti-103), B8 (M, wild caught Nagarhole Park, India, from S. J. O'Brien, ID Pti-104), and B9 (M, wild caught Nagarhole Park, India, from S. J. O'Brien, ID Pti-105).

Panthera tigris altaica: S1 (F, Omaha Zoo, ID 6067, SB 2895), S2 (F, Philadelphia Zoo ID 101571 via Brookfield Zoo ID 26-F-3,4; SB 3110), S3 (F, Minnesota Zoo ID MN5467 via Brookfield Zoo ID 26-G-5; SB 3124), S4 (M, Minnesota Zoo ID MN5466 via Brookfield Zoo, ID 26-H-5,6; SB 3123), S5 (M, Wildlife Conservation Society ID 861078; SB 3009), S6 (M, Wildlife Conservation Society ID 861079; SB 3010), S7 (F, Wildlife Conservation Society ID 721344; SB 845), S8 (F, Omaha Zoo ID 5684; SB 2645), S10 (M, Omaha Zoo ID 4530; SB 2430), S11 (F, Omaha Zoo ID 3976; SB 2393), S12 (F, San Diego Zoo ID 173268 via S. J. O'Brien ID Pti-66; SB 762), S13 (F, S. J. O'Brien, ID Pti-111), S14 (F, Dallas Zoo ID 812821 via S. J. O'Brien, ID Pti-85; SB 1852), and S15 (M, S. J. O'Brien ID Pti-106).

Panthera tigris sumatrae: Su1 (M, Omaha Zoo ID 5448; SB 527), Su2 (F, Omaha Zoo ID 5228; SB 380), Su3 (F, San Diego Zoo ID 589111; SB 753), Su4 (M, San Diego Zoo ID 103497; SB 312), Su5 (M, San Diego Zoo ID BK-8), Su6 (M, London Zoo SB 592), Su7 (M, San Diego Zoo ID 587362 via S. J. O'Brien ID Pti-95; SB 718), Su9 (M, National Zoo ID NZP110519 via S. J. O'Brien ID Pti-109), and Su10 (M, San Diego Zoo ID 687610 via S. J. O'Brien ID Pti-82).

Panthera tigris corbetti: C1 (F, Cincinnati Zoo ID 190014; SB 6), C2 (F, Cincinnati Zoo ID 190015; SB 18), and C3 (F, San Diego Zoo ID 592048; SB 28).

Panthera leo: Brookfield Zoo ID 34-L-8.

Historically, breeding records for zoo animals have varied in quality, but modern-day studbooks of tigers are considered to have high reliability as far as breeding histories are concerned (R. Tilson, pers. comm.). All samples used in this study are assumed to be 'pure bred' representatives of their subspecies and no individual was used if there was the slightest evidence it might be of hybrid origin (for example, large numbers of 'Bengal' tigers in zoos are descendants of hybrids). Because mitochondrial DNA was being assayed, care was taken not to use samples that were known to share the same maternal lineage. For those individuals with studbook numbers it was possible to trace lineages back four or five generations, and thus the independence of many of the individuals could be reasonably assessed. For those specimens represented by studbook numbers, the majority could be traced back to the wild, but at most only to country of origin several generations ago. We discovered no example in which an individual's ancestry involved an unexpected geographic origin.

The sample sizes used here are less than ideal, but for certain questions larger sample sizes are unnecessary (assuming correct identification of the subspecies). If two or more subspecies lack diagnostic variation relative to each other, increasing their sample sizes will not change that pattern of variation and identify them as being distinct. If, however, two or more subspecies are distinct using given sample sizes, then it may be that an increase in sample size for any of them will reveal the existence of polymorphism at the diagnostic sites, thus falsifying the hypothesis of their taxonomic distinctness. The current geographic distribution of tigers is 'artificial', in the sense that they have been eradicated from many areas, thus except possibly through the use of

museum collections much of the original geographic range cannot be sampled. Osteological and skin collections may prove useful in the future for genetic analysis, but were not employed in this study.

Isolation of mtDNA and nuclear DNA fragments

The original goal of this study was to use sequence variation in the mitochondrial cytochrome *b* gene to assess taxonomic distinctness in tigers. During the early phases of this study, however, various primer pairs used in PCR regularly produced two sets of fragments whose sequences, while clearly cytochrome *b*-like, did not match each other and extensively confounded analysis within and among individual tiger subspecies. Because of the prevalence of insertions of mammalian mitochondrial genes into the nuclear genome (Zhang & Hewitt, 1996; Lopez, Yuhki *et al.*, 1994; Zischler *et al.*, 1995; Van der Kuyl *et al.*, 1995), including cytochrome *b* (Smith, Thomas & Patton, 1992; Collura & Stewart, 1995), it was suspected that tigers, like other cats (Lopez *et al.*, 1994), had a nuclear insert and that our primers were amplifying both products.

We decided to undertake long PCR amplifications in an attempt to isolate true mitochondrial sequences on the working assumption that only a small portion of the mitochondrial genome had been transferred to the nucleus. This strategy was successful because of significant length differences in the amplified products. One of these long PCR amplifications, designed to amplify the complete cytochrome *b* gene and the adjacent control region, produced two fragments: (a) a long fragment (sized at about 2800 bp on agarose gels) that is interpreted as being mitochondrial in origin, and (b) a shorter fragment (about 2500 bp) that is interpreted as being a mitochondrial-like nuclear insertion (Fig. 1). Both fragments contain a complete cytochrome *b*-like (1140 bp) sequence; the remainder of both fragments was determined to be, respectively, a long and short control region, along with adjacent tRNAs (evidence for this interpretation is given in the Results).

Once it was confirmed that long PCR was able to separate mitochondrial and nuclear fragments, all subsequent analyses on individual tigers to ascertain their genetic distinctness were undertaken with amplifications using only the long cytoplasmic mitochondrial fragment as a template.

Long-PCR and PCR of Cytochrome *b*

For long PCR from whole blood, a hot-start PCR was performed using the TaKaRa LA PCR system to amplify DNA between the 5' end of cytochrome *b* and the 3' end of the control region. Standard 50 µl reactions were overlaid with mineral oil and subjected to one to three preheating cycles in a block thermal cycler to liberate DNA and inactivate Taq DNA polymerase inhibitors. Each preheat cycle was ramped to 95° C, held for 5 min, ramped down to 80° C, and held for 5 min. On completion of the last cycle, the tubes were kept at 80° C while 0·5 µl (2·5 U) of TaKaRa LA Taq DNA polymerase were added under the oil and mixed. The reactions were then subjected to 35 PCR cycles. Aliquots (5 µl) of these reactions were separated on 1% low

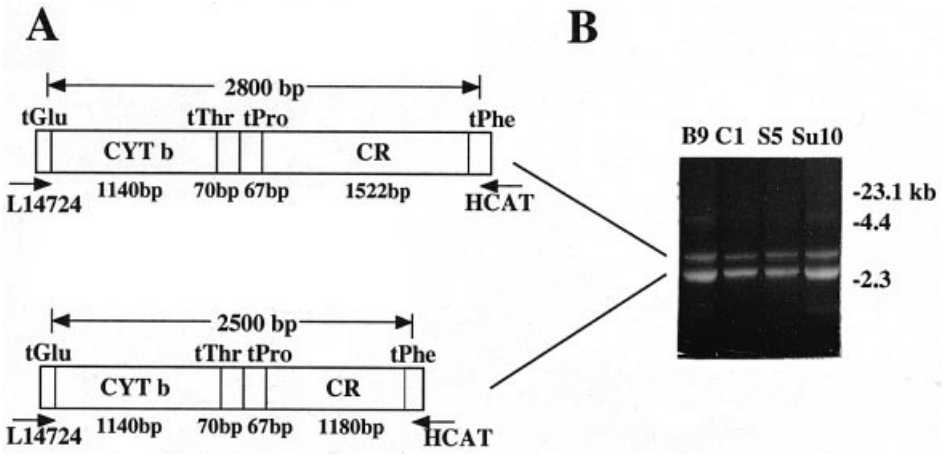

Fig. 1. Discrimination of mitochondrial and putative nuclear cytochrome *b* genes. (a) Long-PCR of tiger blood using primers (L14724 in $tRNA^{Glu}$, HCAT in $tRNA^{Phe}$) spanning the cytochrome *b* gene, the control region (CR), $tRNA^{Thr}$, and $tRNA^{Pro}$ produces two products, one approximately 2800 bp in length (top) and a shorter product approximately 2500 bp (bottom). Reamplification of the entire cytochrome *b* and control region from the former yields sequences interpreted to be typical of feline mitochondrial cytochrome *b* and control regions, whereas reamplification from the shorter product results in complete cytochrome *b*-like and control region sequences interpreted here as being nuclear in origin. It was determined that compared to the normal feline mitochondrial sequence (Lopez *et al.*. 1996), approximately 342 bp of sequence is missing from the control region and its variable repeats of the nuclear copy (see Fig. 2). (b) The two products from the long PCR are found in all tigers sampled, separated here on a 1·0% agarose gel. Samples shown include Bengal 9 (B9), Indochinese 1 (C1), Siberian 5 (S5), and Sumatran 10 (Su10).

melting-temperature agarose gels. The gel-purified long fragment of mtDNA was used as a template for amplification of internal subfragments, including the cytochrome *b* gene, that could be used for direct sequencing. For subsequent capillary PCR, plugs of amplicons were excised from these agarose gels with sterile pipettes and melted at 72° C in 250 µl H_2O for 15 min, and then reamplified in an Idaho Technologies Air Thermo-Cycler (see Nunn & Cracraft, 1996). PCR product was cleaned with the BIO 101, Inc. Geneclean II System and was resuspended in 18 µl H_2O.

Primers used for long PCR, for amplification of internal subfragments, and for sequencing, are listed in Table 1.

Automated sequencing

DNA was sequenced using dye-terminator chemistry in an Applied Biosystems 377 automated sequencer following the manufacturer's recommendations. Sequences were edited and assembled with Sequencher 3.0 (Genecodes) software. The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF053018–AF053058).

Restriction digests

A 463 bp fragment of cytochrome *b* containing a mutation in Sumatran tigers that forms the recognition site for *Spe*I was amplified with primers TL1 and TH2 (see Table 1) following procedures as described above. The products were digested with *Spe*I (Sigma) in 50 µl reactions: 10 µl capillary PCR reaction, 9 U Spe I, Palette Buffer™ Yellow (10 mM Bis-Tris-propane HCl, pH 7.0, 1 mM dithiothreitol, 10 mM $MgCl_2$) (Sigma). Reactions were incubated at 37° C for 2 hours and analysed on 2% agarose–ethidium–bromide gels.

Table 1. Primers used for PCR amplifications and sequencing

Primer name	Sequence
L14724[a]	5' -CGAAGCTTGATATGAAAAACCATCGTTG-3'
L14919	5' -ACTAGCAATACACTACACAGCAGA-3'
H14955	5' -GAGTCAGCCATATTGGACGTCTCGGC-3'
H15149	5' -AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'
TL1/L15120	5' -CCCCTCAAAAAGACATTTGGC-3'
TH1/H15152	5' -GTGCTATTGTTTACGGTCATGGC-3'
TL15162	5' -TTACCATGAGGCCAAATATC-3'
H15356	5' -CTGCATGAATTCCTATTGGGTTGTTTGATCC-3'
TL2/L15504	5' -TTCTCACCAGACCTATTAGGAGACCC-3'
L15507	5' -CCAGACCTCCTAGGAGACCCAGA-3'
TH2/H15537	5' -ATTCGGGCTTAATATGGGGAGGGG-3'
H15561	5' -ATAGCGTAGGCGAATAGGAAGTATC-3'
L15771	5' -ACATGAGTCGGAAGCCAACC-3'
H15915	5' -AACTGCAGTCATCTCCGGTTTACAAGAC-3'
L15995/tRNA^{Pro}	5' -ACCATCAGCACCCAAAG-3'
HCAT[b]	5' -ATTTTCAGTGTCTTGCTTT-3'

[a] Numbers of primers are referenced to the human sequence (Anderson *et al.*, 1981); L and H refer to the L- and H-strand, respectively; T refers to primers designed specifically for tigers.
[b] Primer at the 3' end of the control region used in long PCR (from Hoelzel *et al.*, 1994).

Phylogenetic analysis

Sequences were easily aligned by eye. The presence of fixed molecular markers among tiger subspecies was established by inspection of aligned mtDNA sequences. Discovery of cladistic structure and tree building were undertaken with PAUP 3.1 (Swofford 1992). A branch and bound search was performed in order to guarantee recovery of all most parsimonious trees. Previous molecular studies have confirmed lions as members of the felid genus *Panthera* and close relatives of tigers (Johnson *et al.*, 1966; Collier & O'Brien, *1985*; O'Brien *et al.*, 1987; Wayne *et al.*, 1989; Janczewski *et al.*, 1995), therefore lion sequences were used to root the tiger tree. When mitochondrial and nuclear insertion sequences were included in the same analysis, the domestic cat (*Felis catus*) sequence (Arnason *et al.*, 1995; Lopez, Cevario & O'Brien, 1996) was used as the root.

RESULTS

Evidence for the isolation of mtDNA sequences and presumptive nuclear insertions

The interpretation of the mitochondrial and nuclear origins for the fragments of Fig. 1 is supported by at least five lines of evidence. The long fragment of Fig. 1 is interpreted as being mitochondrial in origin for the following reasons.

(i) The cytochrome *b* sequences amplified from this fragment are virtually identical to previously published sequences of tiger cytochrome *b* (Janczewski *et al.*, 1995; Arnason *et al.*, 1995),

(ii) Variation among tigers is exceedingly low (less than 1%) which is consistent with the findings of previous studies using other techniques to measure genetic distance (O'Brien *et al.*, 1987; Goebel & Whitmore, 1987; Wayne *et al.*, 1989),

(iii) Its length (about 2800 bp) matches that predicted for a complete cytochrome *b* gene (1140 bp), normal feline tRNA sequences, and a feline control region that is comparable in length to, and can be homologized with, that of the domestic cat (about 1500–1600 bp; Lopez, Cevario *et al.*, 1996) and corrected for the length of the tiger repeat sequence (Hoelzel *et al.*, 1994).

(iv) Phylogenetic analysis (described below) leads to the inference that the rate of molecular evolution of the tiger mitochondrial sequences has been significantly divergent relative to the presumptive nuclear insert, which would be predicted from previous studies (Arctander, 1995; Zhang & Hewitt, 1996).

(v) The control region sequences from this fragment, particularly the repeat sequences, match the profile for feline and tiger mtDNA (Hoelzel *et al.*, 1994).

The short fragment is interpreted as being nuclear in origin for the following reasons.

(i) Mitochondrial sequences of tigers are known to occur in the nuclear genome (Johnson *et al.*, 1996).

(ii) The cytochrome *b* sequences from this fragment are phylogenetically closest to lion mitochondrial

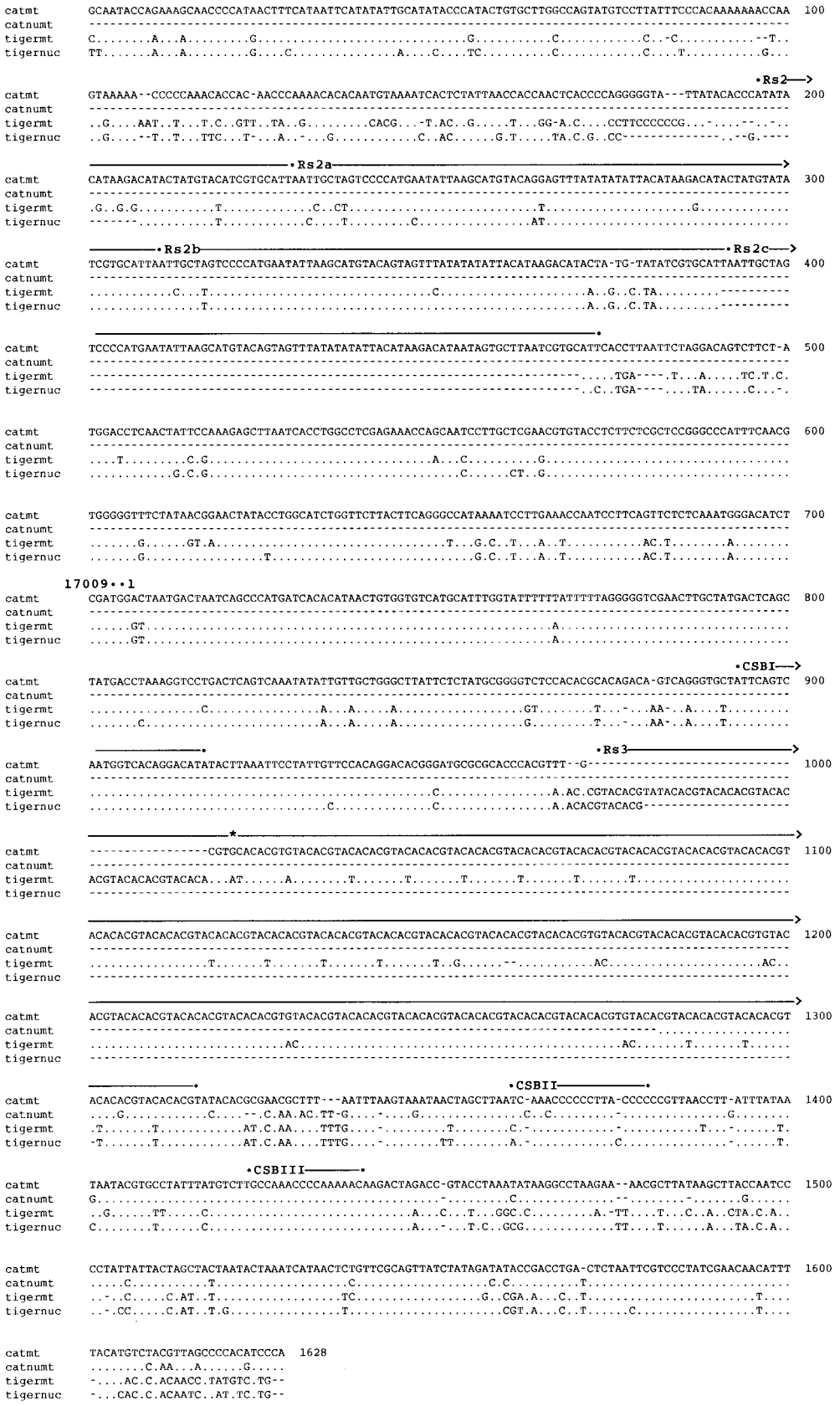

Fig. 2. Aligned sequences of a Siberian tiger (S12) mitochondrial control region and a Siberian (S12) nuclear insert control region, along with those of a domestic cat and the cat *Numt* insert (Lopez, Cevario *et al.*, 1996). Gaps are indicated by a dash. Boundaries of the Rs2 repeat motifs, conserved sequence blocks CSB I, CSB II, and CSB III, as well as the Rs3 repeat sequence are shown for the cat and follow Lopez, Cevario *et al.* (1996). The asterisk in the Rs3 repeat marks the first of the 37 repeats of the 8 bp motifs hypothesized to be homologous between cat and tiger (see Fig. 3).

sequences (described below) and are thus interpreted as retaining a primitive sequence pattern relative to the mitochondrial sequences, a predicted result for nuclear insertions.

(iii) The control region of this fragment lacks the repeat sequences found in the feline and tiger mitochondrial genome (Hoelzel *et al.*, 1994), which has also been found to be the case in the nuclear inserts of the domestic cat (Lopez, Yuhki *et al.*, 1994; Lopez, Cevario *et al.*, 1996).

The control region sequences provide some of the strongest evidence for the identity of the two fragments shown in Fig. 1. The sequences inferred to be mitochondrial have the Rs3 repeat motifs typical of mammals and especially of felids (Hoelzel *et al.*, 1994). Likewise, the 2500 bp fragment inferred to be a nuclear insert lacks those repeat motifs as well as those of Rs2c (Fig. 2).

The sequences for the control region of a Siberian tiger (S12) and its nuclear counterpart are shown in Fig. 2, aligned against those of the domestic cat (Lopez, Cevario *et al.*, 1996). With the exception of several small hypervariable regions, the mitochondrial and nuclear-insert control regions are easily alignable. The control region of the tiger cytoplasmic mitochondrial fragment is 342 bp longer than its nuclear counterpart, most of the difference being the presence or absence, respectively, of repeat sequences in the Rs3 site (Hoelzel *et al.*, 1994; Lopez, Cevario *et al.*, 1996). Within both cat and tiger, the mitochondrial and nuclear insert sequences have greater sequence similarity to one another than they do to their homologues in the other species. Thus, cat mitochondrial sequences exhibit 7·3% sequence similarity to the cat *Numt* (gaps treated as missing); the comparable distance for the two tiger sequences is 6·3%. Cat mitochondrial and *Numt* sequences, on the other hand, are 12·7% and 17·5% from their homologues in tiger.

The tiger and cat cytoplasmic control regions are very similar in sequence pattern and easily aligned. The most significant differences lie in the absence in the tiger of the third Rs2 repeat and the addition of six 8 bp motifs at the beginning of the Rs3 repeat sequences, which are missing in the cat (Fig. 3).

The evidence shown here also supports the interpretation that the tiger nuclear insert is different from the *Numt* insert reported for the cat (Lopez, Yuhti *et al.*, 1994; Lopez, Cevario *et al.*, 1996). In contrast to the tiger insert, the cat insert lacks sequences that are homologous to cytochrome *b*, $tRNA^{Thr}$, $tRNA^{Pro}$, as well as to 1223 bp of the left (5') side of the control region. The cat *Numt* sequence lacks sequence homology to most of the control region and begins only toward the end of the Rs3 repeat sequence (Lopez, Cevario *et al.*, 1996). The tiger nuclear insert, in contrast, spans the entire control region, but has three major sequence blocks missing when compared to the cytoplasmic mitochondrial sequence: (i) an approximately 24 bp block near the beginning of repeat sequence Rs2, (ii) a 79 bp block encompassing nearly all of the third Rs2 repeat (also missing in the tiger cytoplasmic control region), and (iii) a 322 bp block encompassing nearly all of the Rs3 repeat sequence. *Numt* is restricted to a single chromosome, D2; the extent and location of the tiger insert has yet to be determined.

Cytochrome *b* sequence variation among tigers

Comparisons were made among the published tiger cytochrome *b* sequence (Arnason *et al.*, 1995) and those of 34 newly determined cytochrome *b* sequences, including nine Sumatrans, three Indochinese, eight Bengals, and 14 Siberians. Pairwise comparisons across the cytochrome *b* gene indicate that individual tigers exhibit less than 1·0% sequence difference from one another, with there being only 17 variable sites (1·49% of the gene) distributed among 11 distinct haplotypes (Fig. 4a). These findings corroborate the results of previous studies that tigers are very similar genetically (O'Brien *et al.*, 1987; Goebel & Whitmore, 1987; Wayne *et al.*, 1989).

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                                                                    *------>
cat         -------- -------- -------- -------- -------- -----CGT GCACACGT GTACACGT ACACACGT ACACACGT
tigermt     GTACACGT ATACACGT ACACACGT ACACACGT ACACACGT ACACACGT ATACACGT ATACACGT ATACACGT ATACACGT

cat         ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT
tigermt     ATACACGT ATACACGT ATACACGT ATACACGT ACACACGT ACACACGT ACACACGT ACACACGT ATACACGT ATACACGT

cat         ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT GTACACGT ACACACGT ACACACGT GTACACGT ACACACGT
tigermt     ATACACGT ATACACGT ATACGCGT ACA--CGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT

cat         ACACACGT ACACACGT GTACACGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT GTACACGT ACACACGT
tigermt     ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT ACACACGT ATACACGT

cat         ACACACGT ACACACGT ACACACGT
tigermt     ATACACGT ATACACGT ATACACGT
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Fig. 3. Alignment of the control region repetitive sequence site Rs3 for a Siberian tiger (S12) and its nuclear insert, along with those of the domestic cat and its *Numt* insert (Lopez, Cevario *et al.*, 1996). Gaps are indicated by a dash. The asterisk marks the beginning of the 37 motifs hypothesized to be shared by cat and tiger. Also shown are the six 8 bp motifs of the tiger that are not shared with the cat. Comparison and alignment of the tiger with cat suggest that the Rs3 repeat is made up of only 8 bp repeats rather than 6 and 8 bp as originally described (Lopez, Cevario *et al.*, 1996), but this hypothesis may have to await more detailed comparison with other felids.

(a)

Haplotype	Site 1 2333555677999 0 4682146556501059 8 5768926185469879 9
Published tiger+Su4	TGACTTGCGGCGTTAGA
Su1+Su2+Su3+Su5+Su7+Su9+Su10	.......T.........
Su6	....C..T.........
C1+C2	..TT...T.ATG..G..
C3	..TT...T...ACCGA.
B2	CAT...ATA..AC.G..
B3+B4+S1+S2+S3+S4+S7+S12+S15	..T....T...ACCGA.
B5+B6+B7+B8+B9	..T..C.T...A..G..
S5+S13	..T....T...ACCG.G
S6+S8	..T....T...ACCG..
S10+S11+S14	..T....T...ACCGAG
Lion	.AT..CATAACA.....

(b)

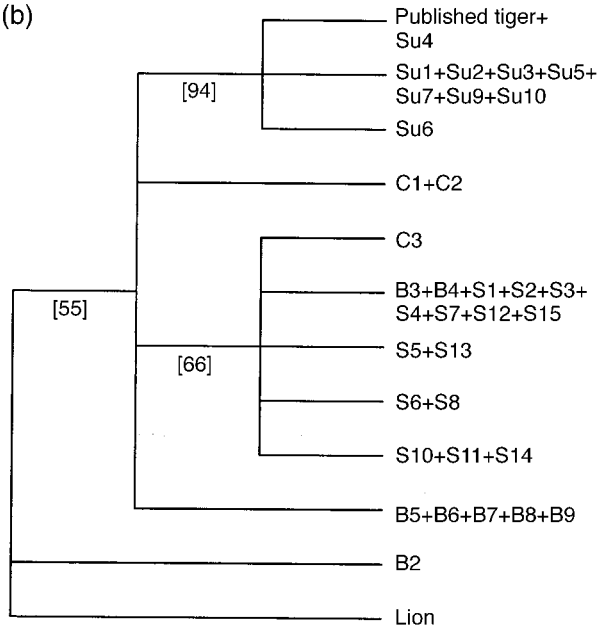

Fig. 4. (a) Variable sites of mitochondrial cytochrome *b* for 11 tiger haplotypes and the lion (Arnason *et al.*, 1995). Three sites (numbers 186, 706, 957) can be used to diagnose Sumatran tigers as a distict taxon relative to other tigers. (b) A strict consensus tree produced by phylogenetic analysis of these 11 tiger haplotypes and using lion to root the tree. A cladistic analysis yielded 25 equally parsimonious trees of 23 steps using the branch and bound option of PAUP 3.1 (Swofford, 1992). The three Sumatran tiger haplotypes cluster together thus confirming the distinctness of Sumatrans as a separate taxonomic unit. The numbers in brackets indicate the percentage of bootstrap support produced by 200 bootstrap replicates.

Diagnosability and phylogenetic analysis of tiger cytochrome *b* sequences

Inspection of the 17 variable sites (Fig. 4a) reveals that the three Sumatran tiger haplotypes share three diagnostic markers relative to other tigers: (i) a transversion change from T to A at site 186, (ii) a transition change from A to G at site 706, and (iii) a transition difference A–G at site 957 that is interpretable as a derived change on some phylogenetic trees but a primitive retention on others. These three markers also support the hypothesis that the unidentified published tiger sequence (Arnason *et al.*, 1995) was derived from a Sumatran tiger or, at least, a Sumatran maternal lineage (its origin is unknown; U. Arnason, pers. comm.). The two haplotypes of the Indochinese tiger share a diagnostic transition change from C to T at site 228. No diagnosable sites were found for Siberian or Bengal tigers, and some individuals of both forms were found to share the same haplotype.

The results of a phylogenetic analysis of the 11 tiger haplotypes with lion added as the root of the tree are shown in Fig. 4b. Because of the scarcity of phylogenetically informative sites, the analysis yielded 25 equally parsimonious trees of 23 steps in length. As indicated by the strict consensus tree, the three Sumatran haplotypes clustered together in each of the most parsimonious trees. The two haplotypes of the Indochinese tiger, however, did not cluster, as the C3 haplotype was united with four other haplotypes consisting of all the Siberians and two Bengals. One Bengal (B2) was placed at the base of all parsimonious trees. This individual retains (or secondarily acquired) three primitive nucleotides (sites 67, 366, 558) shared with lion but which are not found in any other tiger sampled.

Cytochrome *b* sequence variation in the lion

The new lion sequence of cytochrome *b* is very similar to the one previously determined (Arnason *et al.*, 1995). They differ by nine substitutions (seven transitions, two transversions), which represents a 0·79% sequence difference. The lion sequences differ from the 35 tiger sequences at 111 to 117 base positions, or 9·74 to 10·26%; of these changes, there are seven or eight transversion differences (0·61–0·70%) depending upon the pairwise comparison. These sequences are interpreted as being mitochondrial in origin because of their typical length and sequence patterns to one another, as well as to other mammalian cytochrome *b* genes. A third partial sequence, however, previously published as being lion mitochondrial cytochrome *b* (Janczewski *et al.*, 1995), exhibits significant sequence differences from the two discussed here and, moreover, has an 18 bp deletion relative to other cats. This partial sequence, which does have a general cytochrome *b*-like sequence pattern, is also significantly different from putative lion nuclear cytochrome *b* sequences currently under study in our laboratory; this published sequence is likely to be nuclear in origin and not mitochondrial.

Presumptive cytochrome *b* nuclear insertions in tigers

A complete cytochrome *b*-like gene was amplified and sequenced from the short 2500 bp fragment of long PCR experiments (Fig. 1) for one Sumatran tiger (Su1) and one Bengal (B7). Both are virtually identical and differ from each other only by two transition substitutions;

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                                     11111111111111111222222222222222222222233333333333333333333333334444444444444444444444445555555555555
                 3345566666777889000112222345566788900111122333444466788901223344455555666677888990011223344555667777788901235556667888
                 192470367935801968947015751132986984706792512403491492810214392581245703692514737254706281403958124796981959128145415
-----------------------------------------------------------------------------------------------------------------------------------------
TigerSu (mt)     ATTTTCACGCTCTTTTTACGGCTTTCTCACTAACTCTCCGACCGATATCTCCTAGCACCCAATCGTGGCAGTGCGTCCATTAAGTTGCCCCGACTTGACCTGAGTTGCCCGTCGCCC
Sul (nuc)        .CCC.TTT.T...KC.CG.AAT..CTC..T.TT.CT.....TTA.....C..C..T...T.G.TAC.A..AC...........AC.A.T..AG...A...CA..CC..T.A..A...
B7 (nuc)'        .CCC.TTT.T...KC.CG.AAT..CTC..T.TT.CT...A.TTAG....C..C..T...T.G.TAC.A..AC...........AC.A.T..AG...A...CA..CC..T.A..A...
Lion (mt)        G..C.TT.ATC...C...TAAT..CTC..TC.T.C...T...TA..GC.C..C..T.TT...CTAC.ATG.CATA......GGAC.TT.TTA..CC.G....GACC..T.A..A...
Felis catus      ...CCT.....TC.CCCT.AC.CC..CTG...CT..CT..TTTA.C..TCTTCGATG...G.CTACAA..A.A.ACTTGCC...CCA..T...TCC..TA.A..GCAGTTACTATGA

                                                                                                   11111111111111111111111
                 55666666666666666666666667777777777777777777888888888888888888888899999999999999999999999990000000000000000000111
                 990001133333445567788999900001344555667788888000111234455567788889900001111223455666777889999011333344455677888889223
                 240365834569284732848036756894578369280403489479069840925873415685801692567476347369019270369547235814503549023670581

TigerSu (mt)     CATCACTGGTGCCCCACCTTTACCTAGCCCCATACTTCTCCCCATTGCCGTCCCTTCTATCTCTATACTCTCTGCCAAAAACACTTCTGACTGATTACCCTTGCCTCTCTCCCGCCT
Sul (nuc)        ..CTGTCA..AT...G..CCCG.TC.A..A...G.C.....T..CC.G...T...C..G.TC.C.....A..C..TT..GG.G..CTC.G...G.CCTTTC.AT.C.CT..TTA...
B7 (nuc)         ..CTGTCA..AT...G..CCCG.TC.A..A...G.C.....T..CC.G...T...C..G.TC.C.....A..C..TT..GG.G..CTC.G...G.CCTTTC.AT.C.CT..TTA...
Lion (mt)        .GCT.T....AT.TT.TTC.C..TCTA.TA....TCCTC.TT.GCCA.T.CTT.......T.TC.....A......TGT..T....TCAG...G......CCA..C.C..TT.A.TC
Felis catus      T..T.TCTACA.T....TCCC.TTC..TTATGC..AC..A.TT...A.TAC..TCCTCGC..ACCCGACACTAAT.C......TC..CAGTCA.C...TGCCA.GCTCAC...ATAC
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Fig. 5. The 242 variable sites of mitochondrial cytochrome *b* for the published sequences of the Sumatran tiger and lion (Arnason *et al.*, 1995), the putative nuclear sequences of Su1 and B7 reported in this study, and the mitochondrial cytochrome *b* gene sequence of the domestic cat (Lopez, Cevario *et al.*, 1996) for comparison with a non-pantherine felid.

neither possesses an insertion or deletion nor do they have a chain-termination codon within the sequence. These two sequences, however, are very distinct from the mitochondrial cytochrome *b* sequences of Su1 and B7 and those of all other tigers examined (Fig. 5), differing by 8–9 transversions (0.75%) and 92–98 transitions (8·10–8·60%), depending on the pairwise comparison. Among all 34 tigers, amino acid replacements are found at seven codon positions (1·8%), whereas both these putative nuclear sequences exhibit replacement differences from all tiger mitochondrial sequences at 12 positions (3·2%).

Phylogenetic analysis of mitochondrial and presumptive nuclear insertions

The hypothesis that these anomalous cytochrome *b*-like sequences are nuclear is strengthened by a phylogenetic analysis of all 242 variable sites for the 11 tiger mitochondrial haplotypes, two lion sequences, two nuclear sequences, and the domestic cat added as an outgroup. The analysis yielded 70 equally parsimonious trees of 292 steps (Fig. 6). Relationships among the tiger mitochondrial clones are nearly identical to those shown in Fig. 4b, and Sumatran tigers remain a distinct lineage relative to all other tigers. Using the domestic cat mitochondrial cytochrome *b* sequence to root the tree, the analysis results in a strongly supported relationship between the putative tiger nuclear sequences and the lion mitochondrial sequences, but not between the two sets of tiger sequences.

DISCUSSION

Evolutionary change in the tiger mitochondrial and nuclear genomes

Interpretations about the age of the cytochrome *b* gene transfer to the nucleus depend on a correct understanding of relationships within the pantherines and other cats,

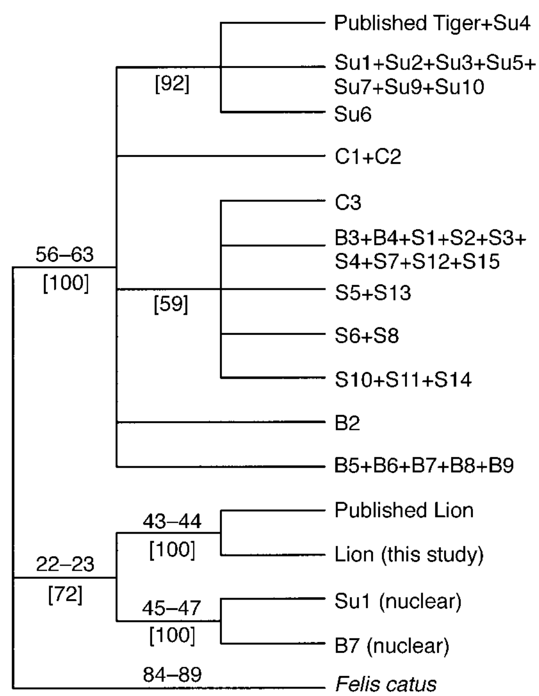

Fig. 6. A strict consensus tree produced by phylogenetic analysis of mitochondrial cytochrome *b* sequences for the 11 tiger haplotypes, the published lion (Arnason *et al.*, 1995), and the lion sequence determined in this study, the two putative nuclear cytochrome *b* sequences reported here, and using the mitochondrial cytochrome *b* sequence of the domestic cat (Lopez, Cevario *et al.*, 1996) as a root for the tree. A cladistic analysis yielded 70 equally parsimonious trees of 292 steps using the branch and bound option of PAUP 3.1 (Swofford, 1992). The numbers in brackets below the branches indicate the percentage of bootsrap support produced by 200 bootstrap replicates. The numbers above the branches indicate the range of branch lengths on the 70 equally parsimonious trees. In all equally parsimonious trees the Sumatran tigers are clustered together and separated from all other tigers, and the two putative nuclear sequences are united to the mitochondrial lion sequences.

as well as knowledge about relative rates of molecular change between mitochondrial and nuclear genomes. Relationships within pantherines are not completely understood, with different data sets giving different patterns of relationships (Collier & O'Brien, 1985; Janczewski *et al.*, 1995; Johnson *et al.*, 1996), and some of these may themselves be influenced by confounding mitochondrial and nuclear sequences (Janczewski *et al.*, 1995).

The pattern of relationships shown in Fig. 6 suggests that the nuclear insert is at least as old as the split between tigers and lions. Yet, if the nuclear insert has been evolving much more slowly than its mitochondrial counterpart, as other studies indicate happens (Zhang & Hewitt, 1996), then the nuclear insert may be much older. Comparative analysis of other species will be necessary to resolve this.

The origin of Sumatran tigers

The findings of this study are consistent with the hypothesis that Sumatran tigers were isolated, and then subsequently differentiated, on Sumatra after a rise in sea-level created that island approximately 6000 to 12 000 years ago (Seidensticker, 1986). The small amount of genetic divergence between Sumatrans and other tigers reflects that recent age of origin and is paralleled by the slight genetic differences reported in the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) and mainland rhinoceros populations (Amato *et al.*, 1995), also thought to be isolated from one another by the same sea-level rise. Given the diagnostic status of Sumatran tigers, our data provide no evidence to suggest interbreeding with the mainland after isolation and differentiation.

Units of analysis: systematics *versus* conservation?

Within systematics, few debates have been as long standing as that surrounding what, if anything, is a species. That debate has only increased in intensity over the past decade or so as phylogeneticists weighed-in with varying opinions on how species should be defined (see citations to this literature in Nixon & Wheeler, 1990; Davis & Nixon, 1992; Davis, 1996). This controversy has now spilled over into conservation biology (e.g. Ryder, 1986; Cracraft, 1991, 1997; Geist, 1992; Rojas, 1992; Moritz, 1994*a*, *b*; Vogler & DeSalle, 1994; Barrowclough & Flesness, 1996; Sites & Crandall, 1997; among others), and the issue becomes, what are we to protect or manage?

Despite many biologists' indifference-bordering-on-disgust attitude toward the debate over species concepts, the stakes are high indeed because numerous consequences follow from how we might subdivide nature taxonomically (Cracraft, 1989, 1997) – and those consequences are not restricted only to systematics. They affect how biologists view patterns of diversity and endemism, how variation is apportioned between and among groups, and in some cases how population sizes of various taxonomic entities might be determined. And, for conservation biology, species concepts have their own set of consequences: determining units for captive breeding and management, specifying units to be protected under law, or regulating trade in endangered taxa.

Much of the recent controversy within conservation biology seems to be between those who prefer to view the problem of units-in-nature from a position of formal taxonomy and those who wish to avoid formal taxonomy altogether and apply another set of terms considered to be relevant to conservation action. Among the latter are 'evolutionary significant units (ESUs)', 'management units', 'stocks', and other such expressions. Leaving aside the difficulties conservation biologists have had in deciding what constitutes an 'evolutionary significant unit', there are more fundamental reasons for abandoning such language (Cracraft, 1997): ESUs have no status within taxonomy, and it is formal taxonomy that historically underpins the language of biodiversity science and is now recognized within legal frameworks (Geist, 1992). The benefits of a formal, universal language for taxonomic diversity cannot be overestimated as it is backed-up by centuries-old scholarship, tradition, and widely accepted rules of procedure; the same thing cannot be said for this new terminology. Moreover, the objectives underlying the attraction of terms such as ESUs are met – as the proponents of ESUs admit – by several formal species concepts, particularly by a phylogenetic species concept.

Adopting a formal species concept will not automatically result in less confusion over units than will the different uses of the term ESU. Even within the phylogenetic species concept there are nuances of definition that might – although most of the time will not – imply different boundaries to taxonomic units. And, there is always the question of the amount and quality of evidence being brought to bear on a given problem. But despite this, it will be to the long-term advantage of conservation biology to operate within the framework of formal taxonomy rather than adopt a proposed terminology that does not offer any clear scientific advantages, and which at some point must be reconciled with formal taxonomy if there are to be lasting legal outcomes.

The ESU concept was introduced, and has gained in support, because it was recognized early that the biological species concept (BSC) could not provide a consistent terminological solution to the units-in-nature problem and thus to conservation biology (Ryder, 1986; Moritz, 1994*a*; Vogler & DeSalle, 1994; Barrowclough & Flesness, 1996; Cracraft, 1997). Conservation biologists cannot use biological species themselves as a unit of conservation because too many biological species contain multiple, differentiated, and often geographically isolated taxa in their own right. Subspecies, moreover, are not easily used because although some are distinct, geographically localized units, others are arbitrary subdivisions of continuously distributed geographic variation and are not distinct units. Moreover, the line between subspecies and biological species is fuzzy, depending often on an arbitrary assessment of degree of

difference. Recent discussions over the applicability of species concepts to conservation have generally supported the notion that some form of the phylogenetic species concept (PSC) is most appropriate for conservation biology (Cracraft, 1991, 1997; Vogler & DeSalle, 1994; Amato *et al.*, 1995; Barrowclough & Flesness, 1996).

Many systematists and conservation biologists have recognized the fact that phylogenetic species are largely what conservation biologists mean by evolutionary significant units (Cracraft, 1991, 1997; Vogler & DeSalle, 1994; Amato *et al.*, 1995; Zink & Kale, 1995; Barrowclough & Flesness, 1996). That being the case, and for the other reasons noted above, it makes sense for conservation biology to substitute the formal taxonomic language provided by the phylogenetic species concept for the informal ESU. Phylogenetic species are basal, diagnosably distinct taxa; that is, they are comprised of one or more populations that share a combination of characters that distinguish them from other such units (see Nixon & Wheeler, 1990; Davis & Nixon, 1992; Davis, 1996; and Sites & Crandall, 1997 for detailed discussions on diagnosing phylogenetic species). Given the data available, phylogenetic species cannot be subdivided into other diagnosable units, hence the notion of their being basal, or terminal, taxa. The PSC is an evolutionary lineage concept, and a populational concept, thus it cannot be applied to single individuals unless those individuals are clonal organisms; within biparental organisms the PSC is applicable to populations, not individual organisms – in other words, it is a taxic concept. Designating a population, or group of populations, as a phylogenetic species is a scientific hypothesis, whose merit is to be adjudicated on evidence; as that evidence changes over time, so too can the conclusion of specific recognition. Under the PSC subspecies are dispensable since if they are diagnosably distinct they would be recognized as a separate phylogenetic species, and if they are not, they would be clustered with other similar populations within another phylogenetic species.

There are other species concepts – evolutionary species concept (Wiley, 1978), cohesion species concept (Templeton, 1989), and alternative versions of the PSC (Donoghue, 1985; Frost & Hillis, 1990) – that will often, but not always, result in the recognition of similar species-level units as the PSC described above. Each of these differ in fundamental ways from the biological species concept, and each has closer correspondence to the idea of an ESU than does the BSC, and thus they are to be preferred. Whichever species concept is applied to any particular conservation problem, and whether one wants to recommend a formal taxonomic name to reflect the results of that analysis, what is important is to adopt a framework that will permit the identification of all diagnosably distinct populations. The latter are evidence of historically distinct units in nature that may require conservation action.

Diagnosable units within tigers

The sequence data offer support for two diagnosably distinct taxa of extant tigers: Sumatran tigers, on the one hand, and all mainland forms, on the other. Evidence for the distinctness of Sumatran tigers is provided by the presence of three unique nucleotide differences, one of which results in an amino acid replacement, as well as by a phylogenetic analysis that strongly isolates Sumatrans from other tigers (Fig. 4). These data do not preclude the existence of genetic markers for *amoyensis*, *tigris*, *corbetti*, or *altaica*. Indeed, the three *corbetti* individuals share a derived transition (site 228; Fig. 4a), but other characters from cytochrome *b* cluster C3 with Bengals and Siberians, and thus the hypothesis that *corbetti* is distinct cannot be supported. While fixed differences in mtDNA can serve as historical markers for delineating taxa, the absence of such markers cannot be taken as evidence that other (unsampled) diagnostic character variation, whether genetic or morphological, does not exist.

The observation that one subspecies of tigers is diagnosably distinct based on derived characters, whereas the other subspecies are not, raises the issue of how to treat this variation taxonomically. Under the traditional biological species concept (Mayr, 1963) tigers have been classified as a single species with multiple subspecies, but this nomenclature cannot represent accurately the historical pattern of variation or the diagnostic status of the different subspecies implied by the evidence presented in this study. One solution within the context of the BSC might be to recognize a single species with two subspecies. Under the phylogenetic species concept (Cracraft, 1989; Barrowclough & Flesness, 1996; Nixon & Wheeler, 1990; Davis & Nixon, 1992; Davis, 1996), in contrast, two species-level taxa would be recognized, *P. sumatrae* and *P. tigris*. Whereas *P. sumatrae* can be shown to be an unambiguous taxonomic unit based on shared derived characters, *P. tigris* presently cannot (although the possibility exists that subsequent studies will show that *P. tigris* can be subdivided). This taxonomic conclusion is consistent with known biogeography: the Sumatran tiger is an island isolate, and populations of mainland *tigris*, *corbetti*, *amoyensis*, and *altaica* apparently had a continuous distribution but are now only recently disjunct because of human-caused habitat destruction (Mazak, 1981).

Conservation implications

Genetic tests for diagnostic taxa are important elements in identifying and regulating illegal trade and managing *in situ* and *ex situ* breeding programs. The above results permit us to devise a simple assay for Sumatran tigers. Primers TL1 and TH2 amplify a 463 bp product encompassing the Sumatran diagnostic site 706, which represents a change from A to G and results in an amino acid replacement. This change also creates a *Spe*1 cleavage site between bases 703 and 704. After digestion, frag-

ments of 351 bp and 112 bp are easily identified on agarose gels without the need for sequencing.

The results of this study have several important implications for captive breeding programs. Space for captive breeding of tigers is limited (Maguire & Lacy, 1990) and heavy biased in favor of Bengals and Siberians. Sumatran tigers are the third most commonly represented form in zoos, but they occupy only about 25% of the space devoted to Siberians and 17% of the space given over to Bengals. Because Sumatrans are diagnosably distinct relative to populations on the mainland, an effort should be made to increase the proportion of space devoted to their captive breeding. Care should be exercised, moreover, to guard the genetic integrity of Sumatrans and to assay Sumatrans of uncertain parentage. Although the test described above is capable only of identifying Sumatran maternal lineages, it nevertheless may help resolve certain questions of suspect parentage.

At the same time an effort should be made to use these results to increase political support for conservation of Sumatran tigers in the wild. Indonesia now can claim patrimony to a distinct taxonomic entity and has an added responsibility to maintain viable wild populations. Efforts to increase the extent and effectiveness of protected areas take on an heightened sense of urgency. Moreover, the fact that Sumatrans are taxonomically and genetically distinct can be used in increasing educational awareness of the importance of conservation programs for Sumatran tigers.

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