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Evolutionary analysis of a large mtDNA translocation (*numt*) into the nuclear genome of the *Panthera* genus species

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Abstract

Translocation of cymtDNA into the nuclear genome, also referred to as *numt*, has been reported in many species, including several closely related to the domestic cat (*Felis catus*). We describe the recent transposition of 12,536 bp of the 17 kb mitochondrial genome into the nucleus of the common ancestor of the five *Panthera* genus species: tiger, *P. tigris*; snow leopard, *P. uncia*; jaguar, *P. onca*; leopard, *P. pardus*; and lion, *P. leo*. This nuclear integration, representing 74% of the mitochondrial genome, is one of the largest to be reported in eukaryotes. The *Panthera* genus *numt* differs from the *numt* previously described in the *Felis* genus in: (1) chromosomal location (F2—telomeric region vs. D2—centromeric region), (2) gene make up (from the *ND5* to the *ATP8* vs. from the *CR* to the *COII*), (3) size (12.5 vs. 7.9 kb), and (4) structure (single monomer vs. tandemly repeated in *Felis*). These distinctions indicate that the origin of this large *numt* fragment in the nuclear genome of the *Panthera* species is an independent insertion from that of the domestic cat lineage, which has been further supported by phylogenetic analyses. The tiger cymtDNA shared around 90% sequence identity with the homologous *numt* sequence, suggesting an origin for the *Panthera numt* at around 3.5 million years ago, prior to the radiation of the five extant *Panthera* species.
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Keywords: Big cats; Mitochondrial DNA; Nuclear insertion; *Numt*; *Panthera* genus; Pseudogene; Tiger

1. Introduction

Nuclear DNA sequences that are homologous to the mitochondrial genome, often referred to as *numts* (pronounced "new-mights," Lopez et al., 1994), have been reported in numerous organisms, including more than 60 animal and plant species (reviewed in Bensasson et al., 2001). Most of the described incidences of *numt* are of short fragments of less than 600 bp with varying degrees of similarity with cymtDNA (Zhang and Hewitt, 1996a; Herrnstadt et al., 1999) and the process of integration has been often associated with non-homologous recombination (e.g., Roth et al., 1985; Henze and Martin, 2001). In humans, the genome sequence database has provided a broad view of the extent of mtDNA transfer, has

Abbreviations: *ATP8*, ATP synthase subunit 8; bp, base pairs; *Cyt b*, cytochrome *b*; *COI*, cytochrome c oxidase subunit I; *COII*, cytochrome c oxidase subunit II; cymtDNA, cytoplasmic mitochondrial DNA; *CR*, control region; kb, kilobase(s); FISH, fluorescence in situ hybridization; MYA, million years ago; mtDNA, mitochondrial DNA; *ND1*, NADH dehydrogenase subunit 1; *ND2*, NADH dehydrogenase subunit 2; *ND5*, NADH dehydrogenase subunit 5; *ND6*, NADH dehydrogenase subunit 6; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; *16S*, 16S ribosomal RNA; *12S*, 12S ribosomal RNA.

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facilitated the identification of transfer mechanisms, and has illuminated the evolutionary dynamics of *numts* (Mourier et al., 2001; Tourmen et al., 2002; Woischnik and Moraes, 2002; Hazkani-Covo et al., 2003; Mishmar et al., 2004; Ricchetti et al., 2004). The incorporation of mtDNA sequences into the human nuclear genome has probably been a continuous evolutionary process, with, by some estimates, at least 612 integrations (Woischnik and Moraes, 2002). However, the incidence of novel *numt* insertions may be lower, since mtDNA-like sequences may also result from duplication after insertion into the nucleus (Tourmen et al., 2002; Bensasson et al., 2003; Hazkani-Covo et al., 2003). Most human *numt* segments encompass less than 5% of the mtDNA, and in only three instances exceed 70% of mtDNA.

Whole genome sequences of other mammals will continue to elucidate the evolutionary dynamics of *numts* outside of humans (Pereira and Baker, 2004; Richly and Leister, 2004). However, full genome drafts of other mammals will be limited primarily to model organisms of biomedical, taxonomic or phylogenetic interest (O'Brien et al., 2001). Therefore detailed characterizations of *numts* among closely related species will be necessary to provide additional insights into the characteristics of mitochondrial pseudogenes, including the study of their evolutionary histories and their distribution and abundance across species (Bensasson et al., 2001; Pons and Vogler, 2005).

There have been two documented cases of *numt* that have been reported in the Felidae family. The first consisted of the translocation of 7.9 kb of the mitochondrial genome into the domestic cat (*Felis catus*) nuclear genome (Lopez et al., 1994). This large segment is tandemly repeated 38–76 times on cat chromosome D2. The second case of *numt* in the Felidae family was first described in *Panthera* genus species based on mtDNA RFLP data (Johnson et al., 1996) and later by sequence analysis (Cracraft et al., 1998). Here we characterize the structure and evolutionary history of the *Panthera numt* fragment by (i) determining its chromosomal location in all the *Panthera* genus species (tiger, *P. tigris*; snow leopard, *P. uncia*; jaguar, *P. onca*; leopard, *P. pardus*; and lion, *P. leo*), (ii) comparing large portions of the *numt* and cymt sequences in one *Panthera* species (the tiger), and (iii) employing phylogenetic and coalescence analyses to assess the evolutionary history of these *numt* and cymt segments in species of the genus *Panthera*.

2. Materials and methods

2.1. DNA isolation, amplification, cloning and sequencing

To facilitate the characterization of the *Panthera numt*, three distinct DNA fractions [total (t), nuclear (n), and cytoplasmic mitochondrial (cymt)] were purified from 1.5 g of frozen liver from tiger (Pti065), snow leopard (Pun086), jaguar (Pon011), leopard (Ppa021), and lion (Ple181). The tDNA (mixture of nDNA and cymtDNA) was extracted from tissue according to standard procedures (Sambrook et al., 1989; Lopez et al., 1994). The nDNA fraction was purified using sucrose gradient DNA extraction methods (Bernatchez and Dodson, 1990) and the cymtDNA was purified using the Wizard Miniprep kit (Promega, Beckman et al., 1993). Four regions of the mtDNA genome were amplified in each of the fractions: (i) a portion between the *ND5* gene and the *CR* (primers ND5F-U/CRR-U), (ii) the *CR* segment (primers CRF-U/CRR-U), (iii) a portion from *16S* to *ND2* (primers 16SF-U /ND2R-U), and (iv) the segment from *ND2* to *ATP8* (primers ND2F-U /ATP8R-U) (Fig. 1; Table 1). RFLP analysis was performed on these segments using several restriction enzymes (*Bam*HI, *Hind*III, *Eco*RI, *Xho*I, etc.) to test for differences in banding patterns between cymt and *numt*. The PCR products for the *CR* and *16S-ND2* segments, which exhibited different size lengths in the nDNA and cymtDNA fractions, were cloned and sequenced to unambiguously distinguish the cymt and *numt* products. PCR products were purified using Microcon PCR (Amicon). Cloning was carried out using Zero Blunt TOPO PCR Cloning kit (Invitrogen). The smallest *CR* PCR products were purified after agarose gel electrophoresis and subcloned using pCR-Blunt II-TOPO cloning vector (Invitrogen). Positive clones of cymt and *numt* were confirmed by comparison with the RFLP patterns. The clones were sequenced using Bigdye Terminators Cycle Sequencing Kits (PE Applied Biosystems) and run on an ABI-377 automated sequencer. Based on cymt/*numt* mismatches, a series of *numt*- and cymt-specific primers were designed for long-range PCR, allowing a more extensive sequencing and analysis of the tiger cymt and *numt* (Table 1). *Numt* and cymt strand-specific primers were designed in highly variable sections or for variable sites using the virtual PCR program Amplify-2.53 (Engels, 1997). Additionally, cymt and *numt* portions of the *16S*, *ND1* and *ND2* genes were amplified, cloned and sequenced for all the five *Panthera* species.

2.2. Cytogenetic inference of the Panthera numt location: FISH mapping

The location of *numt* in the nuclear genome of all the *Panthera* species was determined by FISH. A 2.6 kb mtDNA PCR probe (Fig. 1), generated from the purified cymtDNA fraction, was labeled with biotin-11 dUTP (Sigma) by nick translation (Brigati et al., 1983) in the five *Panthera* species, as well as the domestic cat. The final probe size was verified on a 1.2% gel with appropriate markers. Metaphase spreads were prepared by standard cytogenetic techniques (Modi et al., 1987). FISH was performed as described in (Lichter et al., 1990). Briefly the metaphase spreads were denatured in 70% formamide 2XSSC in an 80 °C oven for 90 s and dehydrated in cold ethanol series, 70–90–100%, for 3 to 5 min in each step. 400 ng of labeled probe and 10 ug of salmon sperm carrier DNA were resuspended in 50% formamide-10% dextran sulfate-2XSSC and denatured for 10 min at 75 °C. The denatured probe cocktail was layered on the denatured metaphase chromosomes. Following 48 h of incubation at 37 °C, post-hybridization washes, and treatment with blocking solution, the hybridized biotin labeled probe was detected by fluorescein isothioscyinate (FITC) conjugated avidin DCS (5 mg/ml—Vector labs). Fluorescence signals were captured as gray scale images using a Zeiss Axioskop epi-fluorescence microscope equipped with a cooled CCD (charged coupled device) camera

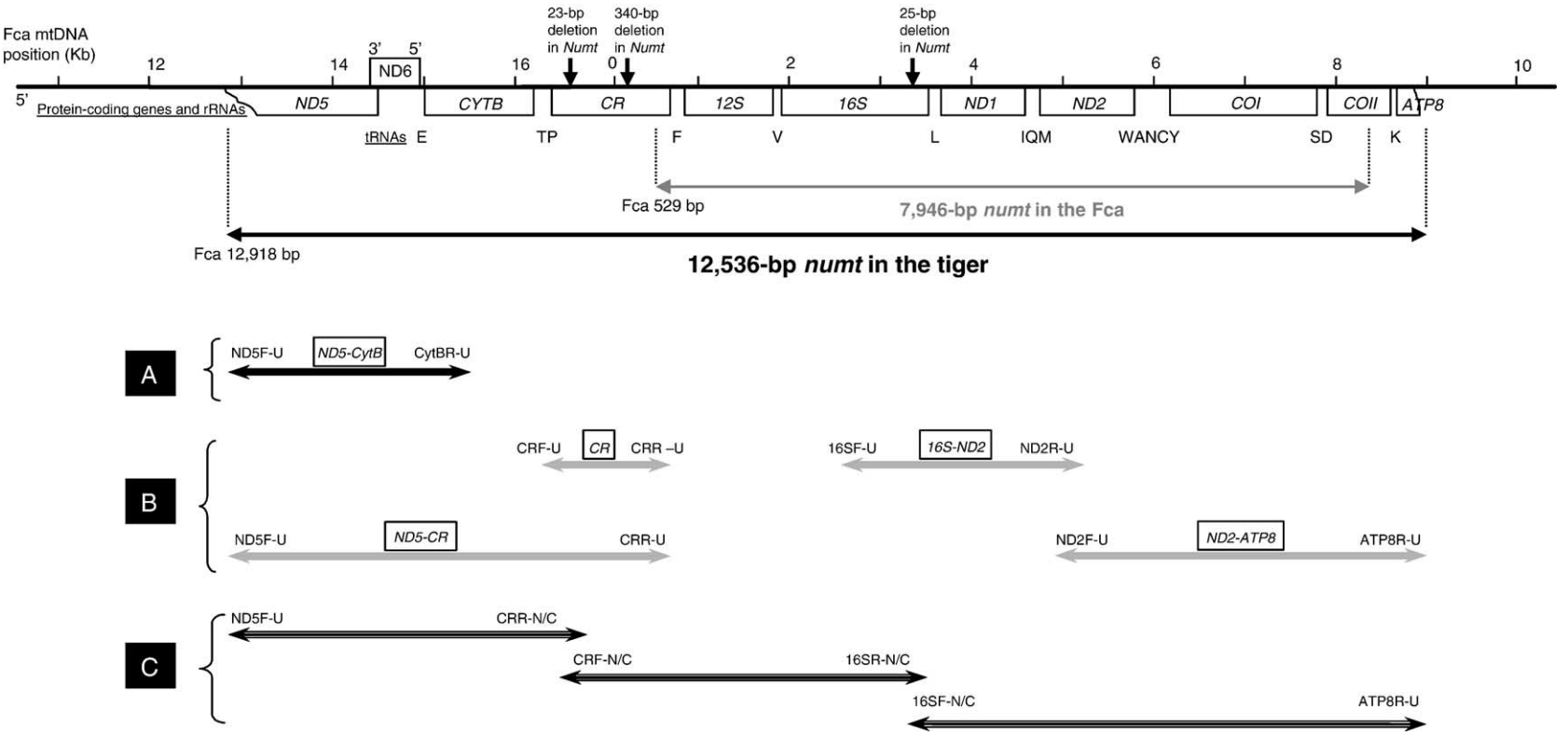

Fig. 1. Schematic diagram of the relative positions of *Panthera numt*. The scale bar (in kilobase) corresponds to the domestic cat (Fca—*Felis catus*) mtDNA complete sequence (Lopez et al., 1996) aligned with the *Panthera numt* described in this study. The Fca *numt* is represented for comparison. Protein-coding genes and rRNAs are indicated in boxes. Individual capital letters correspond to the 17 tRNAs. The arrows and numbers over the *CR* and *16S* represent gaps between the cymt and *numt* sequences in the tiger. Fragments amplified from cymt or *numt* portions are represented by lines and arrow lines with primer names labeled at the 5′ and 3′ ends and primer sequences (Table 1). (A) A 2.6 kb mtDNA probe was generated by PCR and used for FISH mapping to locate the *numt* in the *Panthera* species. (B) Four segments were amplified using universal primers from three DNA fractions (tDNA, nDNA, and cymtDNA) of *Panthera* species and examined by RFLP (see Fig. 2). Two segments (*CR*, *16S-ND2*) were cloned and sequenced subsequently to separate cymt and *numt*. (C) Cymt and *numt* tiger sequences were obtained separately using a combination of universal and strand-specific primers designed based upon cymt/*numt* gaps in the *CR* and *16S* regions (Table 1).

Table 1
Primers used to amplify the *Panthera* cymt and *numt* portions surveyed in this study

Primer name	Sequence	Specificity
ND5F-U	5′-GTGCAACTCCAAATAAAAG-3′	*Panthera* sp.
CytBR-U	5′-ATTAATAATTTTGATAAGGGGGTGCGAT-3′	*Panthera* sp.
CRF-U	5′-TCAAAGCTTACACCAGTCTTGTAAACC-3′	Universal
CRR-U	5′-TAACTGCAGAAGGCTAGGACCAAACCT-3′	Universal
16SF-U	5′-ACGACGGCCAGTGTGCAAAGGTAGCATAATCA-3′	*Panthera* sp.
ND2R-U	5′-CAACCCGTTAACCTCGGGTACTCAGAAGT-3′	*Panthera* sp.
ND2F-U	5′-ACTTCTGAGTACCCGAGGTTAACGGGTTG-3′	*Panthera* sp.
ATP8R-U	5′-GCTATGACCGGCGAATAGATTTTCGTTCA-3′	Universal
CRF-N	5′-ACTCCCACAACACAGACGCACAGT-3′	*P. tigris* N
CRF-C	5′-CGTTAATACAGAACACACAACACG-3′	*P. tigris* C
CRR-N	5′-CATTGTGCGTTTGTGTTATGGG-3′	*P. tigris* N
CRR-C	5′-CGTGTTGTGTGTTCTGTAT-3′	*P. tigris* C
16SF-N	5′-CGTTTGTTCAACGACTACCGG-3′	*P. tigris* N
16SF-C	5′-CAAAGTCCTACGTGATCTG-3′	*P. tigris* C
16SR-N	5′-CGTGGACTACTCCGGTAATCG-3′	*P. tigris* N
16SR-C	5′-CAGAACTCAGATCACGTAG-3′	*P. tigris* C

*The meanings of the abbreviations are as follows; U—*Panthera* species specific or universal primer, N—*numt* specific, C—cymt specific, F—forward, R—reverse. The source of universal primers is Kocher et al. (1989), Johnson et al. (1998), or designed from this study, and N, C primers were designed for this study using clones from *CR* and *16S-ND2* gene regions.

(Photomentics CE 200 A) and the Oncor imaging system. Gray-scale images were computer enhanced, pseudocolored, and merged using Oncor Image software. Images of reverse DAPI banded chromosomes were merged with the FITC detected signals allowing for direct visualization of localization, chromosome identification and cytogenetic loci assignment.

2.3. Sequence analyses

Sequences were inspected using SEQUENCHER (Gene Codes Co.), aligned using Clustal-X (Thompson et al., 1997), and further checked by eye. Initial sequence comparisons and measures of variability were performed using MEGA (Kumar et al., 2001). Transition/transversion ratios (Ts/Tv) and the parameter of the gamma distribution of rate variation among sites method of Yang and Kumar (1996) were estimated using PAMP (included in the package PAML 2.0; Yang, 1997). tRNA structure was predicted using the mfold web server (Zuker, 2003). Phylogenetic analyses of the *Panthera* cymt and *numt* sequences were performed in PAUP* 4.0b2a (Swofford, 2001) using three approaches: (i) minimum evolution (ME) heuristic search, using a Kimura two-parameter model and the neighbor-joining tree-building algorithm (Saitou and Nei, 1987) followed by branch-swapping; (ii) maximum parsimony (MP), with an exhaustive search; and (iii) maximum likelihood (ML), incorporating a gamma-corrected HKY85 model with parameters estimated from the data set. Reliability of nodes defined by the phylogenetic trees was assessed using 100 bootstrap replications (Felsenstein, 1985; Hillis and Bull, 1993) in the ME and MP analyses, and with the quartet puzzling method in the ML analysis (PUZZLE 4.0; Strimmer and von Haeseler, 1996). The molecular dating for the *Panthera numt* origin was estimated from the overall genetic distance between tiger *numt* and cymt, applying the equation of Li et al. (1981) whereby the fraction of sequence divergence is: $\delta=(\mu_1+\mu_2)\,t$, where $\mu_1=2.5\times10^{-8}$ substitutions/sites/year for cymtDNA (Hasegawa et al., 1985; Lopez et al., 1997) and $\mu_2=4.7\times10^{-9}$ substitutions/sites/year for nuclear pseudogene distance (Li et al., 1981; Lopez et al., 1997) and t is the time elapsed.

3. Results

3.1. Recognition of the genes involved in the Panthera numt

A detection strategy was devised to identify and isolate potential *numt* fragments based on differences in banding patterns from four distinct PCR products [(*ND5-CR*), (*CR*), (*16S-ND2*) and (*ND2-ATP8*); (Fig. 1)] and RFLP's banding patterns from three DNA fractions (tDNA, nDNA, and cymtDNA isolated from liver tissue; see Material and methods) (Fig. 2). The *CR*-PCR products from the tDNA fraction in all

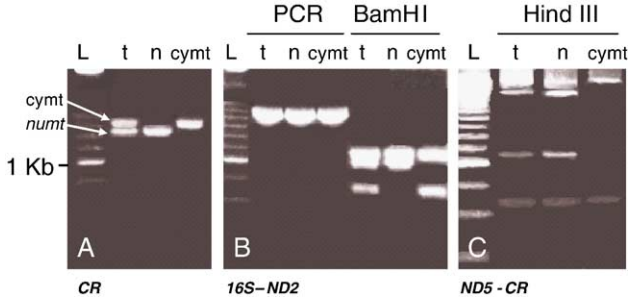

Fig. 2. Differences in the banding patterns from PCR products amplified from total (t), nuclear (n), and cytoplasmic mitochondrial (cymt) *Panthera* DNA fractions from two of the four segments surveyed that showed presence of *numt* copies. The two segments represented in this figure were chosen for depiction due to the clear distinction of *numt* sequences caused by the large deletions in *CR* and *16S*. The banding patterns observed were similar in all *Panthera* species and thus only a single species profile is represented. (A) Control region fragment. (B) Region between *16S* and *ND2* genes followed by restriction enzyme digestion of *Bam*HI. Lane L represents DNA size ladder 250 (BRL, i.e. the brightest band is 1.0 kb and each step represents 250 bp).

the *Panthera* species showed two codominant bands of around 1.7 kb and 1.5 kb, compared with a single band from the purified cymtDNA and nDNA fraction (1.7 and 1.5 kb, respectively) (Fig. 2A). We determined by band pattern and sequence analysis that the 1.7 kb fragment was cymt and that the 1.5 kb fragment was the *numt* copy. *Numt* PCR products were identified also from the three other regions, (*ND5-CR*), (*16S-ND2*; Fig. 2B) and (*ND2-ATP8*) based on different RFLP patterns of *Hind*III and *Bam*HI digestion among the three DNA fractions. These combined results suggested that the *Panthera numt* encompasses a region within the *ND5* to the *ATP8* gene, including eight protein coding genes, two rRNA genes, 17 tRNA genes, and the non-coding *CR* (Fig. 1).

3.2. Chromosomal location of the Panthera numt

A 2.6 kb mtDNA probe including *ND5*, *ND6*, and *CytB* regions (Fig. 1) was hybridized on a metaphase spread of the five *Panthera* genus species and the domestic cat. Strong hybridization fluorescent signals were observed on chromosome F2 at q1.1 in all the *Panthera* species (Fig. 3A to E), but on chromosome D2 at the centromere of the domestic cat (Fig. 3F), as previously described by Lopez et al. (1994).

3.3. Comparative sequence analyses of tiger numt and cymt

Using large deletions in *CR* (25 bp) and *16S* (23 bp) of the *Panthera numt*, we designed strand-specific primers for *numt* and cymt for long-range PCR amplification and sequencing in tiger (Fig. 1; Table 1). Sequences from clones and PCR products were concatenated into a fragment of 12,898 bp for cymt and 12,536 bp for *numt* (GenBank accession numbers DQ151550 and DQ151551) (Fig. 1). The size difference between *numt* and cymt was caused mostly by the 340 bp gap in the RS3 region, a 23 bp gap in the HVS-1 region of *CR*, and a 25 bp gap of the *16S* gene in *numt* (Fig. S1). The *numt* sequence started in the middle of the *ND5* gene position (corresponding to position Fca 12,918 in the domestic cat; Lopez et al., 1996) and almost reached the end of *ATP8* gene (position Fca 8840). This 12,536 bp (~12.5 kb) of tiger *numt* included approximately 75% of the 17 kb mitochondrial genome, as described in the domestic cat (Lopez et al., 1996) The tiger *numt* contains a truncated *ND5* gene (1533 bp), and complete *ND6* (527 bp), *Cyt b* (1143 bp), *12S* (960 bp), *16S* (1545 bp), *ND1* (958 bp), *ND2* (1044 bp), *COI* (1550 bp), and *COII* genes (684 bp), a truncated *ATP8* gene (183 bp), a *CR* sequence (1181 bp) with a large deletion (340 bp) removing most of the RS-3 with the d(CA)-rich 8-bp

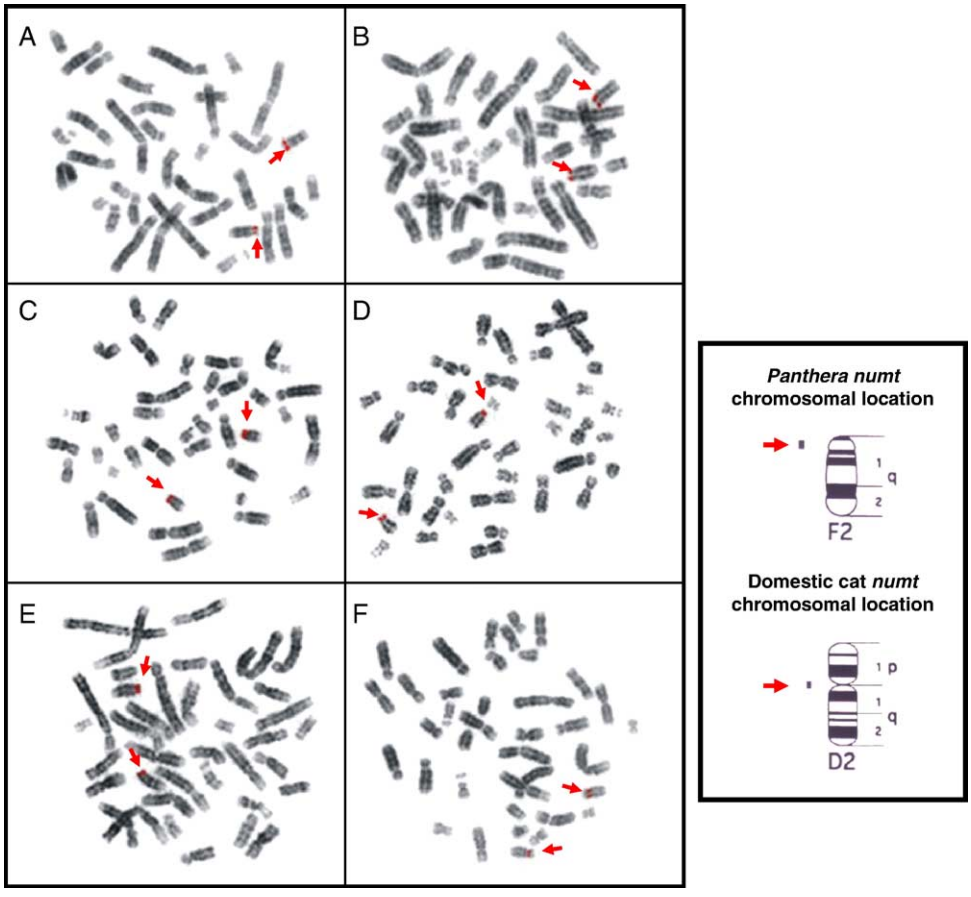

Fig. 3. Image of fluorescent in situ hybridization (FISH) of the metaphase chromosomes for each of the five *Panthera* species and the domestic cat using the probe including the partial sequences from *ND5* and *Cytb* regions (2.6 kb). (A) Tiger, *P. tigris*. (B) Lion, *P. leo*. (C) Jaguar, *P. onca*. (D) Leopard, *P. pardus*. (E) Snow leopard, *P. uncia*. (F) Domestic cat, *F. catus*. Signals revealed on the telomeric region of the chromosome F2 (F2q12) in all the *Panthera* species (A–E) and on the centromeric region of the chromosome D2 (D2p11) in the domestic cat (F).

Table 2
Characterization of the size, similarity, and nucleotide substitution patterns from pairwise comparison of tiger cymt (12.8 kb) and *numt* (12.5 kb) sequences. Stop codons within *numt* were determined after frame shift or indels

Segments	Size (bp)		Changes between cymt and *numt* (bp)		Pattern of substitutions							Pattern of gaps in *numt*		Number of stop codons within *numt*	Percent differences of nucleotides
	cymt	*numt*			Ts		Tv				Ts/Tv ratio	Insertions (bp)	Deletions (bp)		
			Substitutions	Gaps	A-G	T-C	G-C	A-T	T-G	A-C					
Protein coding genes															
ND5	1530	1533	124	11	35	76	4	3	1	5	8.5	7(1bp×3+2bp×2)	4(1bp×4)	1	8.8
ND6	528	527	38	5	14	19	–	1	3	1	6.6	2(1bp×2)	3(1bp×1+2bp×2)	–	8.1
ND1	957	958	71	5	15	52	1	1	1	1	16.8	3(1bp×4)	2(1bp×2)	3	7.0
ND2	1044	1044	84	4	26	46	4	4	–	4	6.0	2(1bp×2)	2(1bp×2)	–	8.4
CytB	1140	1143	94	15	29	59	1	2	1	2	14.7	9(1bp×9)	6(1bp×6)	–	10.5
COI	1545	1550	124	19	48	65	1	2	6	2	10.3	12(1bp×9+3bp×1)	7(1bp×7)	23	9.3
COII	684	684	62	8	27	33	–	–	1	1	30.0	4(1bp×2+2bp×2bp×1)	4(1bp×4)	–	10.2
ATP8	182	183	23	9	10	9	1	2	1	1	3.8	5(1bp×3+2bp×1)	4(1bp×3+2bp×1)	5	17.6
Total	7610	7622	620	76	204	359	12	15	14	17	9.7	44	32	32	9.1
rRNAs															
12S	957	956	19	9	7	9	–	–	–	3	5.3	4(1bp×4)	5(1bp×5)	–	2.9
16S	1575	1545	42	44	13	22	1	4	–	1	5.8	7(1bp×7)	37(6bp×1+1bp×6+25bp×1)	–	5.5
Total	2532	2501	61	53	20	31	1	4	–	4	5.7	11	42	–	4.5
tRNAs															
tRNA-Glu	71	70	–	1	–	–	–	–	–	–	–	–	1(1bp×1)	–	1.4
tRNA-Thr	70	70	4	–	1	2	–	1	–	–	3	–	–	–	5.7
tRNA-Pro	66	66	–	–	–	–	–	–	–	–	–	–	–	–	0
tRNA-Phe	71	75	6	6	3	1	1	–	1	–	2	5(2bp×1bp×3)	1(1bp×1)	–	16.9
tRNA-Val	68	68	–	–	–		–	–	–	–	–	–	–	–	0
tRNA-Leu	75	75	4	–	–	2	–	1	1	–	1	–	–	–	5.3
tRNA-Ile	69	69	2	–	2	–	–	–	–	–	2	–	–	–	2.9
tRNA-Gln	74	74	–	–	–	–	–	–	–	–	–	–	–	–	0
tRNA-Met	69	69	1	–	–	1	–	–	–	1	–	–	–		1.4
tRNA-Trp	69	69	3	4	–	1	–	1	1	–	0.5	2(1bp×2)	2(1bp×2)	–	10.1
tRNA-Ala	69	69	3	–	1	2	–	–	–	–	3	–	–	–	4.3
tRNA-Asn	73	73	3	–	1	1	–	–	1	–	2	–	–	–	4.1
tRNA-Cys	66	66	2	–	1	–	–	1	–	–	1	–	–	–	3.0
tRNA-Tyr	68	66	4	4	–	2	1	–	–	1	1	1(1bp×1)	3(1bp×3)	–	11.8
tRNA-Ser	70	70	3	–	1	2	–	–	–	–	3	–	–	–	4.3
tRNA-Asp	69	70	4	1	2	2	–	–	–	–	4	1(1bp×1)	–	–	7.2
tRNA-Lys	69	69	4	–	1	3	–	–	–	–	4	–	–	–	5.8
Total	1186	1188	43	16	13	19	2	4	4	1	2.9	9	7	–	5.0
Control region	1539	1181	79	378	25	39	2	6	2	5	4.3	10(1bp×7+3bp×1)	368(1bp×5+23bp×1+340bp×1)	–	30
Total	12,867	12,492	803	523	262	448	17	29	20	27	7.6	74	449	32	10.3

[ACACACGT] motif, and full sequences for 17 interspersed tRNAs (Fig. S1).

3.4. Numt and cymt sequence characterization in tiger

The nucleotide composition of tiger *numt* and cymt sequences were similar, 32.31% A, 26.04% C, 15.13% G, and 26.38% T in *numt* compared with 32.34% A, 26.19% C, 15.10% G, and 26.32% T in cymt. *Numt* and cymt shared three different types of genes (rRNA, tRNA, and protein coding) plus the *CR* (Fig. S1). Markedly different patterns of sequence variation were observed between different *numt* and cymt genes, with sequence similarities ranging from 82% in *ATP8* to 100% in three tRNA (Table 2). Sequence variation between *numt* and cymt was due to both base-pair substitutions ($n=803$) and indels ($n=523$ bp). Most of the mutational changes between *numt* and cymt were transitions (710/803=88%) with the highest proportion of transitional changes occurring in the protein coding genes (5635/611=92%) and the lowest in RNAs (83/103=81%). Transitions from T to C were more common than from A to G. To infer whether these genes retained function, sequences from the protein coding genes of cymt and *numt* were translated into amino acid using the mitochondrial and universal genetic codes, respectively. All cymt protein coding gene sequences could be translated into amino acid sequences, but in the *numt* sequences 32 extra stop codons were observed (Figs. S1 and S2). The variable sites between cymt and *numt* in protein-coding genes were not distributed evenly (Fig. S1A), suggesting that conserved segments may lie within the functional domains of the mtDNA proteins, which are more prone to evolutionary constraints. Likewise, in *12S* there were 26 variable sites in the first half from positions 1 to 530 bp and no variable sites from positions 531 to 1027. In the 1575 bp fragment of *16S*, 74 of 82 (90%) variable sites occurred in the first 520 bp (1–520 bp) and the third 500 bp (1040–1575 bp) compared with only 8 variable sites (less than 10%) in the middle, (from 521 to 1039 bp) (Fig. S1B). Seventeen tRNA genes were sequenced in both cymt and *numt* (Fig. S1C). Three tRNA genes (tRNA-Gln, -Pro, and -Val) had identical sequences in both cymt and *numt*. The number of variable sites in the other tRNA genes ranged from one in tRNA-Met to 12 in tRNA-Phe. Average percentage sequence similarity between cymt and *numt* in tRNA genes was 95% and in rRNA 95.5% (Table 2). Lower sequence similarity was observed for the protein coding genes (90.9%) and the *CR* (91%; excluding the 186 bp gap of RS3 region).

3.5. Phylogenetic relationships of the Panthera numts

The phylogenetic relationships of the cymt and *numt* sequences in the five *Panthera* species was investigated using concatenated sequences (1206 bp) from three mitochondrial

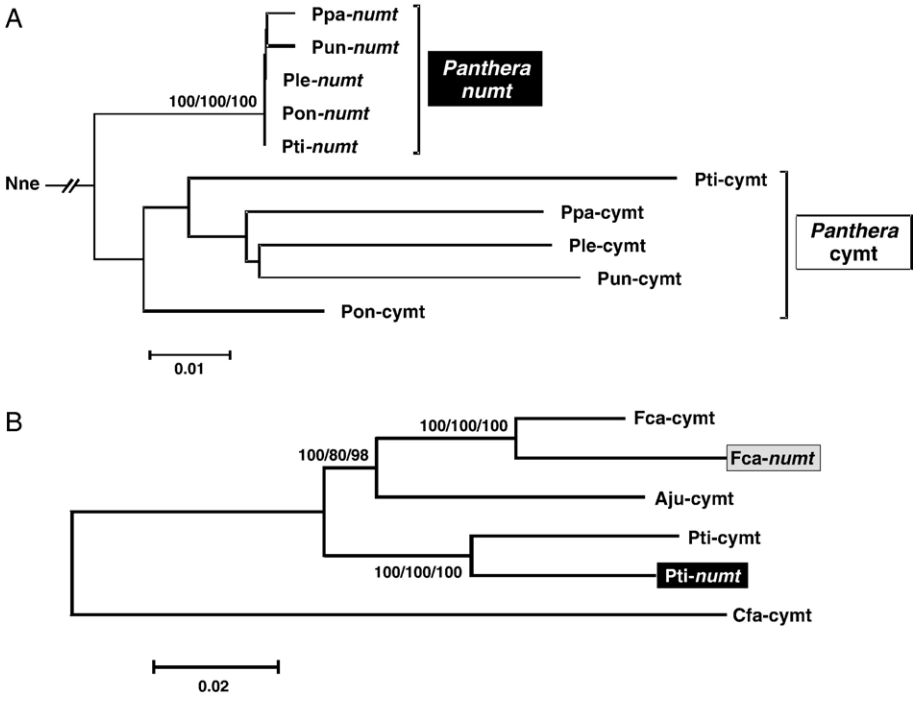

Fig. 4. (A) Phylogenetic minimum evolution tree (Kimura two-parameter) of the five *Panthera* species cymts and *numts* (1206 bp concatenated sequences of the *16S*, *ND1*, and *ND2*). The taxon abbreviation is as follows: Pti—tiger, Pun—snow leopard, Pon—jaguar, Ppa—leopard, Ple—lion, and Nne—clouded leopard (*Neofelis nebulosa*). The rooting of the tree was obtained with the slowest evolving mtDNA fragment (*16S*) to avoid long-branch attraction caused by the high rate of divergence of mtDNA. (B) Phylogenetic minimum evolution tree (Kimura two-parameter) illustrating the relationship between the domestic cat *numt* (Lopez et al., 1996) and the tiger *numt* (this study) (7683 bp alignment). The taxon abbreviation is as follows: Fca—domestic cat, Aju—cheetah (*Acinonyx jubatus*), Pti—tiger, and Cfa—dog (*Canis familiaris*). GenBank accession numbers are as follows: Fca-cymt (***U20753***); Fca-*numt* (***U20754***); Aju-cymt (***NC_005212***); and Cfa-cymt (***NC_002008***). Bootstrap values are placed at each branchpoint for the minimum evolution/maximum parsimony/maximum likelihood phylogenetic analyses, respectively (ME/MP/ML).

genes, *16S* (403 bp), *ND1* (502 bp), and *ND2* (301 bp) (Fig. 4A). The cymt/*numt* specific-amplification of such genes was facilitated by the 23 bp deletion of the *16S Panthera numt*. Two distinct monophyletic clusters, with very strong bootstrap support, defined cymt and *numt* sequences (results were identical considering ME, MP or ML analyses, or each of the single gene sequences analyses). Little internal structure among *Panthera* species was observed in either cymt and *numt* sequences. Cymt sequences showed a five fold faster rate of divergence (average pairwise distance = 0.066 ± 0.006) compared to *numts* (0.013 ± 0.002) (see also Fig. 4A), similar to the pattern observed in *Felis numt* (Lopez et al., 1994). Additionally, the phylogenetic relationships between the domestic cat *numt* (Lopez et al., 1997) and the tiger *numt* (this study) clearly suggest that the two classes of *numts* within Felidae are distinct synapomorphies (Fig. 4B).

4. Discussion

4.1. Origin of the Panthera numt

An independent origin of the *Panthera numt* from that of the domestic cat (Lopez et al., 1994) is strongly supported by its distinct chromosomal location, size, contents, and structure. The *numt* location in all the *Panthera* species was mapped by FISH on chromosome F2 (Fig. 3A to E). However, the signal using the same probe on the domestic cat produced a signal on chromosome D2 (Fig. 3F), as previously described (Lopez et al., 1994). The tiger *numt*, is considerable larger than domestic cat's, with a single unit of 12.5 kb that includes genes from middle of *ND5* to part of *ATP8* subunit (Fig. 1). By contrast, the domestic cat *numt* has a unit of 7.9 kb (with genes from middle of *CR* to *COII*) that is tandemly repeated with 38 to 76 copies, having an overall integrated size of 300 to 600 kb (Lopez et al., 1994). To test for a tandem arrangement in tiger *numt*, we performed inverse PCR with several different primer sets. However, because we did not observe any PCR products, this suggests that the *Panthera numt* is not tandemly repeated and is most likely a single segment on the chromosome F2.

The phylogenetic analysis performed on cymt and *numt* sequences from the five extant *Panthera* species strongly supports a single origin for all these *numts* along the branch leading to the most-recent common ancestor of the genus (Fig. 4A) and that the domestic cat *numt* and the tiger *numt* lineages are distinct synapomorphies within the Cat family (Fig. 4B). Using an overall genetic distance of 10.3% between tiger *numt* and cymt (Table 2), we estimate that *numt* and cymt began to diverge around 3.45 MYA, which would be consistent with the known evolutionary history of the *Panthera* lineage. Analyses of nuclear and mtDNA sequences across all felid species suggests that a common ancestor of the five species of roaring cats diverged from the clouded leopard 5.96 MYA and began to speciate into unique evolutionary lineages 3.47 MYA (O'Brien, 1996; Johnson and O'Brien, 1997; Johnson et al., in press). Overall, our results support the occurrence within the Felidae family of two independent translocations of cytoplasmic mtDNA into the nuclear genome: one in the *Panthera* genus (around 3 MYA) and the other in the domestic cat lineage (around 1.8 MYA; Lopez et al., 1994).

4.2. Numt as a pseudogene: evolution and functional implications

Once mtDNA fragments become incorporated into the nuclear genome, they immediately are exposed to different modes of evolution, which will influence the divergence patterns between the two sequences (Lopez et al., 1994, 1996, 1997). These include lower mutation rates due to nuclear DNA repair, a distinct genetic code, and the possibility of recombination. In addition, *numts* apparently evolve without the functional selective constraints as their mitochondrial counterparts (Gellissen et al., 1983; Perna and Kocher, 1996). The tiger cymt showed a high bias in transitions over transversions, a well-recognized characteristic of mtDNA (Brown et al., 1982) that was not observed for the *numt* sequence (nDNA). The phylogenetic analyses depict the more-rapid rate of cymt divergence among *Panthera*. This is caused by the higher mutation rate of mtDNA, particularly for protein-coding genes (Lopez et al., 1997).

Genes within the tiger *numt* fragment have several characteristics that would preclude these sequences from producing functional gene products. First, in the protein coding genes of *numt*, there are often several termination codons or frame shift mutations in all possible open reading frames (Table 2; Fig. S1A), many of which were caused by differences in the genetic codes between the nucleus and mitochondria (Anderson et al., 1981; Brown, 1985). Second, the *numt 16S* has a large deletion (23 bp), which would appear to disrupt the normal secondary structure (Fig. S1B). Third, two regulatory elements (CSB 2 and 3) of the *CR* that are involved in transcriptional promotion catalyzed by mitochondrial RNA polymerase and trans-activating factors do not function in nuclear genes (Schinkel and Tabak, 1989). The *numt CR* also lacks most of the repetitive segment three (RS-3), which is involved in mtDNA replication and transcription (Fig. S1D). The importance of mtDNA *CR* in the nuclear genome is at least in part dependent on the presence of promoter regions and functional sequences, because as far as is known, the *CR* is only functional with promoter and several protein-binding sites (Chang and Clayton, 1985). Due to the large deletions of the hypervariable segment one (HVS-1) and RS-3, the *numt CR* sequence is presumably not functional. Fourth, all of the cymt tRNA sequences formed typical cloverleaf shapes of class 1 tRNAs (Lewin, 1994). However, some *numt* tRNAs, like for example, tRNA-Thr and tRNA-Tyr, formed imperfect shapes due to several unpaired free-bases that likely cause loss of function (Fig. 5). The differing degrees of similarity among tiger cymt and *numt* genes, specifically the highly conserved rRNAs or invariant tRNA genes contrasted with the more-divergent protein-coding genes and the *CR* (Table 2; Fig. S1C and D), highlight the differential rates of nucleotide substitution among mitochondrial genes relatively to its homologues *numt* molecular "fossils." In the mammalian mitochondria, the

average nucleotide divergence is much lower in rRNA genes relative to protein-coding genes or the *CR* (Lopez et al., 1997).

The maintenance in the function of genes translocated from organelle to nucleus occurred numerous times in evolutionary history, contributing to the compact and economical mitochondrial genomes observe today (Perna and Kocher, 1996). The mammalian mitochondrial genome of 15,000–17,000 bp and 37 coding genes contrasts with the hundreds of nuclear genes that have function in the mitochondria, such as nuclear-encoded members of the citric acid cycle, cytochrome chain, and oxidative phosphorylation pathways. As with *numt*, these nuclear genes, following the Serial Endosymbiosis Theory (Margulis, 1970; Yang et al., 1985), are thought to have originated from the transfer of mtDNA genes to the nucleus, with subsequent duplication and divergence. A reduction in the accumulation of deleterious mutations is a prime benefit for cymt genes that are subsequently located in the nuclear genome (where DNA repair is more efficient). However, functional gene transfers have been documented almost exclusively in plants (e.g., Adams et al., 2002) and green algae (e.g., Perez-Martinez et al., 2000; Funes et al., 2002), suggesting that in animals, where the mitochondrial genetic code differs from the standard code (Wolstenholme, 1992), most *numts* are non functional upon arrival.

4.3. The mtDNA as a reliable molecular marker

The maternal inheritance, cellular abundance, and lack of recombination of the mtDNA have allowed biologists to phylogenetically study many metazoan animal. However, mitochondrial-like DNA sequences in the nuclear genome of many organisms, and their amplification or coamplification during PCR is a recognized complication (Perna and Kocher, 1996; Zhang and Hewitt, 1996a). Because nuclear insertions are paralogs of the authentic mitochondrial sequences, they will confound phylogenetic and population genetic analyses when inadvertently included, especially when using more slowly evolving segments (Arctander, 1995; Collura and Stewart, 1995; Vanderkuyl et al., 1995; Zhang and Hewitt, 1996b). Mitochondrial-like sequences in the nuclear genome can negate the advantages of mtDNA as a molecular marker in population studies. The occurrence of *numt*, as with sequence heteroplasmy, necessitates more-complicated data collection and analysis and in some species, like gorillas that have a large variety of *numt*

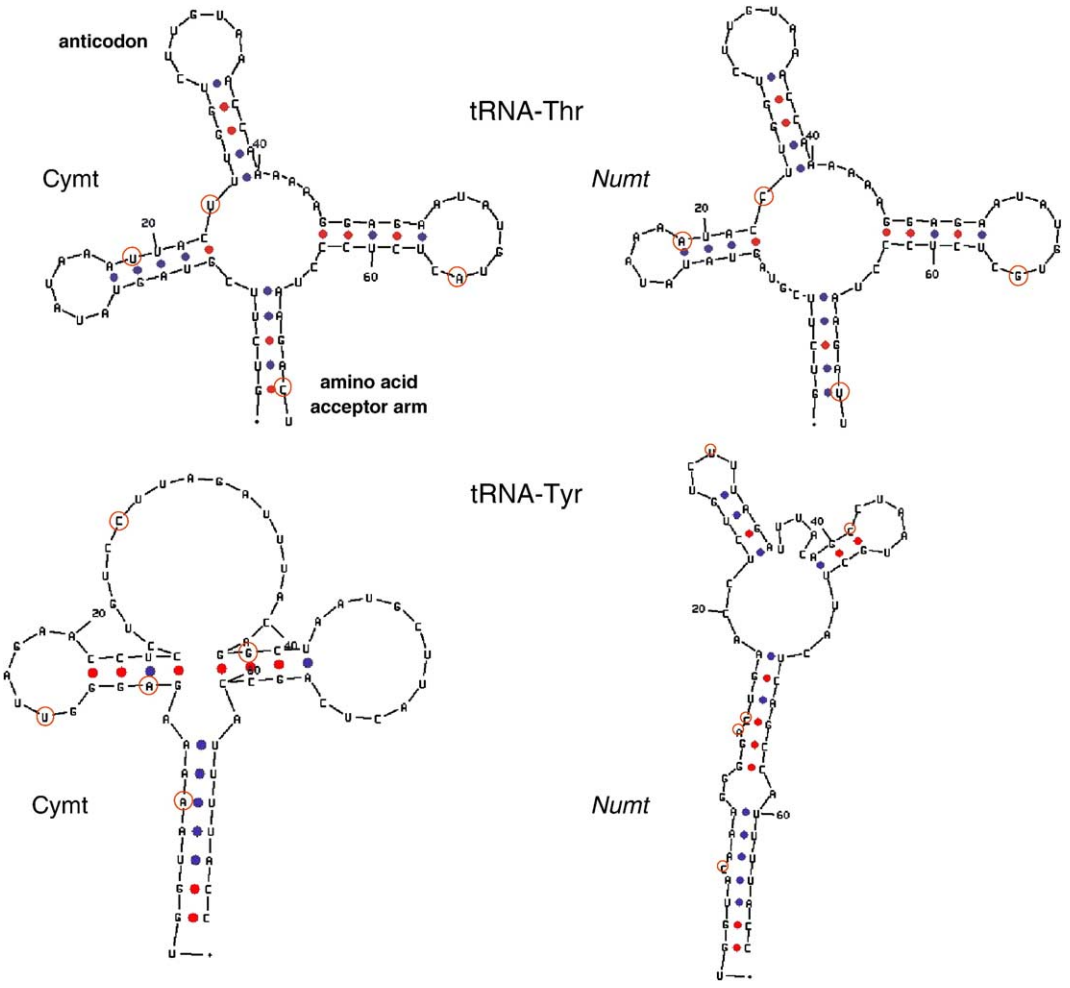

Fig. 5. Proposed secondary structure for tRNA-Thr and tRNA-Tyr based on DNA sequence data from the tiger cymt and *numt*. Dots represent Watson–Crick bonds. Red circles indicate that the nucleotide is variable between cymt and *numt*. Numbers represent the direction of the sequences from 5′ to 3′.

sequences bearing high similarity to cymtDNA, can make analysis of mtDNA impractical (Thalmann et al., 2004).

One implication is that explicit measures need to be taken to authenticate mtDNA sequences generated. Previously reported mtDNA tiger sequences have been incorrectly labeled (Fig. S3). In some cases, the reported gene sequences were mixed sequences of cymt and *numt* (Masuda et al., 1994; Ledje and Arnason, 1996). In another case, sequences were preferentially collected from nuclear copies (Johnson and O'Brien, 1997). The full sequence for both tiger cymt and *numt* is presented here, providing a valuable contribution for research in felids. Such data has greatly facilitated the validation of the matrilineal genealogy of current tiger subspecies (Luo et al., 2004) and certainly will be highly useful for research on the other closely related *Panthera* species. Refined accurate population genetic inferences will represent an effective contribution for the conservation and the management of these endangered cat species.

The relative scarcity of *numts* described in Felidae species to date contrasts with the high frequency of *numts* observed in primates, particularly in humans, as revealed by the human genome database (e.g., Mourier et al., 2001; Tourmen et al., 2002; Woischnik and Moraes, 2002). The prevalence of reported *numts* varies widely among metazoans (reviewed in Bensasson et al., 2001), with human and plant genomes harboring the largest *numt* repertoires (Richly and Leister, 2004). The cat genome project, which was recently included in the Large-Scale Sequencing Research Network, will facilitate more detailed evaluation of the dynamics and extent of *numt* insertions in this Felidae species.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2005.08.023.

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