



Original Article

Genetic Ancestry of the Extinct Javan and Bali Tigers

Hao-Ran Xue*, Nobuyuki Yamaguchi*, Carlos A. Driscoll*, Yu Han*, Gila Kahila Bar-Gal, Yan Zhuang, Ji H. Mazak, David W. Macdonald, Stephen J. O'Brien and Shu-Jin Luo

From the College of Life Sciences, Peking-Tsinghua Center for Life Sciences, Peking University, Beijing 100871, China (Xue, Han, Zhuang, and Luo); the Wildlife Conservation Research Unit, Department of Zoology, University of Oxford, Recanati-Kaplan Centre, Tubney House, Abingdon Road, Tubney, Abingdon OX13 5QL, UK (Yamaguchi, Driscoll, and Macdonald); the School of Life Sciences, Beijing Normal University, Beijing 100875, China (Han); the Koret School of Veterinary Medicine, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Rehovot 76100, Israel (Bar-Gal); the Shanghai Science and Technology Museum, Shanghai 200127, China (Mazak); the Theodosius Dobzhansky Center for Genome Bioinformatics, St. Petersburg State University, St Petersburg 199004, Russia (O'Brien); and the Oceanographic Center, Nova Southeastern University, Ft Lauderdale, FL 33004, USA (O'Brien). Nobuyuki Yamaguchi is now at Department of Biological and Environmental Sciences, University of Qatar, PO Box 2713, Doha, Qatar. Carlos A. Driscoll is now at WWF-India at Wildlife Institute of India, Chandrabani, Dehadun, Uttarakhand 248001, India.

*These authors contributed equally to the study.

Address correspondence to Shu-Jin Luo, at the address above, or email: luo.shujin@pku.edu.cn.

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Abstract

The Bali (*Panthera tigris balica*) and Javan (*P. t. sondaica*) tigers are recognized as distinct tiger subspecies that went extinct in the 1940s and 1980s, respectively. Yet their genetic ancestry and taxonomic status remain controversial. Following ancient DNA procedures, we generated concatenated 1750 bp mtDNA sequences from 23 museum samples including 11 voucher specimens from Java and Bali and compared these to diagnostic mtDNA sequences from 122 specimens of living tiger subspecies and the extinct Caspian tiger. The results revealed a close genetic affinity of the 3 groups from the Sunda Islands (Bali, Javan, and Sumatran tigers *P. t. sumatrae*). Bali and Javan mtDNA haplotypes differ from Sumatran haplotypes by 1–2 nucleotides, and the 3 island populations define a monophyletic assemblage distinctive and equidistant from other mainland subspecies. Despite this close phylogenetic relationship, no mtDNA haplotype was shared between Sumatran and Javan/Bali tigers, indicating little or no matrilineal gene flow among the islands after they were colonized. The close phylogenetic relationship among Sunda tiger subspecies suggests either recent colonization across the islands, or else a once continuous tiger population that had subsequently isolated into different island subspecies. This supports the hypothesis that the Sumatran tiger is the closest living relative to the extinct Javan and Bali tigers.

Subject areas: Molecular systematics and phylogenetics, Conservation genetics and biodiversity

Key words: mtDNA, *Panthera tigris sondaica*, *Panthera tigris balica*, phylogeny

The tiger *Panthera tigris* is among the world's largest species of Felidae. Last century, the wild population declined dramatically from over 100 000 individuals at the beginning of the century to 5000–7000 in the 1990s, to 3000 tigers today (Chundawat *et al.* 2011). Modern tigers occupy 7% of their historical range, which once covered vast regions between the Caspian and Aral Seas, southeastern Russia, and the Sunda islands (Dinerstein *et al.* 2007; Chundawat *et al.* 2011). This decline has been due to habitat loss and fragmentation, prey base depletion and human persecution, and preserving existing wild tiger populations has become a major conservation focus (Dinerstein *et al.* 2007).

The earliest tiger fossils found in northern China and Java (in Indonesia) date back to 2 million years ago (MYA) in the early Pleistocene (Hemmer 1971; Hemmer 1987). Molecular genetic imputation traces all living tigers back to a common ancestor as recent as 72 000–108 000 years ago (Luo *et al.* 2004). It has been speculated that the Toba volcano super eruption in Sumatra approximately 73 500 years ago (Rampino and Self 1992) may have contributed to this recent coalescence for modern tigers (Luo *et al.* 2004).

Mitochondrial DNA sequencing and microsatellite genotyping in tiger conservation (O'Brien and Johnson 2005; Luo *et al.* 2010a) have provided powerful tools to reconstruct phylogeography and demographic history (Mondol *et al.* 2009), assess population genetic status noninvasively (Henry *et al.* 2009; Mondol *et al.* 2009; Sharma *et al.* 2009), validate subspecies ancestry of captive tigers (Luo *et al.* 2008), discern the basis of adaptive traits from a genomic perspective (Cho *et al.* 2013; Xu *et al.* 2013), and inform restoration proposals (Driscoll *et al.* 2011). Advances in ancient DNA technologies have even made it possible to retrieve DNA from degraded historical samples of bones, pelt, and teeth from extinct subspecies (Driscoll *et al.* 2009).

The most important application of genetic techniques has been in resolving taxonomic uncertainty surrounding tiger subspecies (Luo *et al.* 2004, 2010b). Taxonomists have described tigers based on gross morphological characters (size, pelage, and color), habitat, and geographic range and until 2004 recognized 8 subspecies of *Panthera tigris* (Mazak 1981): 1) *P. t. tigris* (Linnaeus, 1758); 2) *P. t. virgata* (Illiger, 1815); 3) *P. t. altaica* (Temminck, 1844); 4) *P. t. sondaica* (Temminck, 1844); 5) *P. t. amoyensis* (Hilzheimer, 1905); 6) *P. t. balica* (Schwarz, 1912); 7) *P. t. sumatrae* (Pocock, 1929); and 8) *P. t. corbetti* (Mazak 1968). Of these, the Bali tiger *P. t. balica*, Caspian tiger *P. t. virgata*, and Javan tiger *P. t. sondaica* went extinct in the 20th century (Chundawat *et al.* 2011). In 2004, *P. t. jacksoni* was recognized on the basis of genetic evidence as a new subspecies (Luo *et al.* 2004) and current tiger taxonomy, informed by molecular genetic evidence, now recognizes 6 living subspecies (Luo *et al.* 2004; Luo *et al.* 2010b; Chundawat *et al.* 2011): Bengal tiger *P. t. tigris*, Amur tiger *P. t. altaica*, south China tiger *P. t. amoyensis*, Sumatran tiger *P. t. sumatrae*, Indochinese tiger *P. t. corbetti*, and Malayan tiger *P. t. jacksoni*. High genetic similarity between the *P. t. altaica* and the extinct *P. t. virgata* has since been revealed (Driscoll *et al.* 2009) and Javan and Bali tigers remain the last 2 putative tiger subspecies whose patterns of genetic diversity, demographic history, and phylogenetic placement in relation to living relatives have not been investigated.

Populations from islands are generally given subspecies status in taxonomy; however, named island subspecies may not carry significant genetic distinctiveness, particularly if gene flow between them occurred recently. During the Pleistocene, sea level fluctuations repeatedly exposed vast areas of the Sunda Shelf, forming land

bridges intermittently among the islands of Sumatra, Java and Bali as recently as the Last Glacial Maximum (LGM, c. 20 000 years ago) and enabling recent population connectivity and possible gene flow (Kitchener and Dugmore 2000; Kitchener and Yamaguchi 2010). However, other studies indicate that the ability of mammals to move across the exposed Sunda Shelf may have been restricted and populations or subspecies have deeper divergence dating back to even MYA (Meijaard and van der Zon 2003; Woodruff and Turner 2009; Luo *et al.* 2014). For example, recent molecular and morphological research have proposed the Javan leopard *Panthera pardus melas* as a distinct taxon that split from other Asian leopards hundreds of thousands of years ago, likely deriving from an ancient leopard population arriving in Java (Uphyrkina *et al.* 2001; Meijaard 2004). Hemmer (1969, 1971) proposed a similar scenario postulating that Javan and Bali tigers represent a relict population from prehistoric tigers during the early to middle Pleistocene and correspond to early tiger fossils found in this region. Craniometrical studies have lent support to this notion suggesting that Javan and Bali tigers are somewhat distinguishable from all mainland and even Sumatran tigers, although there are only minor differences between them (Mazak and Groves 2006).

Settling this aspect of tiger evolution clearly requires genetic studies of museum and private specimens of Bali and Javan tigers. Here, based on gathering and validation of museum samples (Yamaguchi *et al.* 2013) we analyzed the phylogeographic history of Javan and Bali tigers to determine historical genetic diversity and genetic relationships between extinct tigers and extant relatives. Genetic patterns were analyzed jointly with published data from the 6 extant and 1 extinct (Caspian) tiger subspecies (Luo *et al.* 2004, 2008; Driscoll *et al.* 2009; Luo *et al.* 2010b). The results highlight a clearer picture of the evolutionary history and phylogeographic partitioning that have formed modern tiger population structures.

Materials and Methods

Samples

An extensive search was conducted for specimens of extinct tiger subspecies from natural history collections in Asia, Europe, and North America (Yamaguchi *et al.* 2013). Forty-eight samples *P. t. sondaica* and *P. t. balica* were collected for this study with various degrees of certainty in their geographic origin (permissions arranged by N. Yamaguchi, Supplementary Table S1 online). Most samples were small pieces of bones or teeth; bone powder from flake or damaged parts of skulls and dry tissues attached to skulls were sometimes also available. All tissue samples were collected in full compliance with Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) permits issued to the National Cancer Institute, National Institutes of Health (Principal Officer: S. J. O'Brien), by the U.S. Fish and Wildlife Service, and to the College of Life Sciences, Peking University (Principal Officer: S. J. Luo), by the State Forestry Administration of China.

Ancient DNA Procedures

We visually examined the quality and quantity of each sample and selected 33 for downstream analysis (Table 1; Supplementary Table S1 online) following ancient DNA procedures (Cooper and Poinar 2000). Each sample were processed in at least 2 of the 3 independent ancient DNA laboratories (Laboratory of Genomic Diversity, National Cancer Institute, USA, the Hadly Lab at Stanford University, USA,

Table 1. Museum tiger specimens from Sumatra, Java, and Bali ($N = 23$)

Sample code (PKU)	Short code	Museum ^a	Museum ID	mtDNA haplotype code ^b	Geographic origin by record	Subspecies by morphology or geography	Morphology examined by
Nobb0004BO01	N4	Stuttgart	18922	BAL	Bali	Bali	Voucher specimen ^c
Nobb0005BO01	N5	Stuttgart	18923	BAL	Bali	Bali	Voucher specimen
Nobb0009SK01	N9	Amsterdam	13542	SON	Java	Javan	Voucher specimen
Nobb0007SK02	N7	Amsterdam	9174	SON	Java	Javan	Voucher specimen
Nobb0008BO01	N8	Amsterdam	9179	SON	W. Java	Javan	Voucher specimen
Nobb0010BO01	N10	Leiden	264	SON	Java	Javan	Voucher specimen
Nobb0011BO01	N11	Leiden	314	SON	Java	Javan	Voucher specimen
Nobb0017SK01	N17	Leiden	45100	SON	Java	Javan	Voucher specimen
Maza0008TO01	M8	Shanghai	1356	SON	Java	Javan	Voucher specimen
Nobb0020BO01	N20	St Petersburg	5737	SON	Java	Javan	Voucher specimen
Nobb0022BO01	N22	Stuttgart	7628	SON	Java	Javan	Voucher specimen
Nobb0039BO01	N39	Leiden	45095	SON	n/a	Javan?	Yamaguchi <i>et al.</i> (2013)
Nobb0025BO01	N25	Amsterdam	563	SON	n/a	Javan?	Yamaguchi <i>et al.</i> (2013)
Nobb0027BO01	N27	Amsterdam	1829	BAL	Indonesia	Javan?	V. Mazák, before 1980
Nobb0028BO01	N28	Amsterdam	9183	BAL	Indonesia	Javan?	V. Mazák, before 1980
Nobb0040BO01	N40	Leiden	45097	BAL	n/a	Javan?	Yamaguchi <i>et al.</i> (2013)
Nobb0026BO01	N26	Amsterdam	1827	BAL	Indonesia	Javan?	V. Mazák, before 1980
Nobb0034BO01	N34	Leiden	5015 (1417)	SUM1/2/5	n/a	Javan/Sumatran?	N. Yamaguchi
Nobb0032BO01	N32	Leiden	4715	SUM3	n/a	Javan/Sumatran?	N. Yamaguchi
Nobb0047BO01	N47	Leiden	5016 (1422)	SUM3	n/a	Sumatran?	N. Yamaguchi
Nobb0042BO01	N42	Leiden	6715	SUM6/7/8	n/a	Sumatran?	N. Yamaguchi
Nobb0043BO01	N43	Leiden	4700 (1414)	SUMx	n/a	Sumatran?	N. Yamaguchi
Piva0001TO01	NY1	S. Pivar	NY1	SUM6/7/8	n/a	Javan/Bali/Sumatran?	

^aAbbreviations for museums: Stuttgart-Staatliches Museum für Naturkunde in Stuttgart, Stuttgart, Germany; Amsterdam-Zoologisch Museum, University of Amsterdam, the Netherlands (the Amsterdam collection was transferred to Leiden in 2010); Leiden-Nationaal Natuurhistorisch Museum, Leiden, the Netherlands; Shanghai-Shanghai Science and Technology Museum, Shanghai, China; St Petersburg-Zoological Institute, Russian Academy of Sciences, St Petersburg, Russia; S. Pivar-Private collector, New York, USA.

^bVariable sites decoding the mtDNA haplotypes are listed in Table 2.

^cThe first 11 specimens are voucher specimens with confirmed geographic origin from Java or Bali.

and College of Life Sciences at Peking University, China) with extreme precautions taken to minimize contamination risk (Cooper and Poinar 2000). DNA extraction and PCR setup were performed in a room that is physically isolated from the modern DNA facility. The ancient DNA room is equipped with positive air pressure and high-efficiency particulate arrestance filters, preventing extraneous particulate matter from entering. Bleach and UV-light sterilization procedures were conducted regularly to destroy nontarget DNA in the ancient DNA zone, particularly the night before DNA-containing materials were brought into.

DNA extraction was done using a silica-based spin column method modified from protocols described previously (Yang *et al.* 1998; Hadly 2003) in small batches of ≤ 5 samples at a time. For each specimen, 30–100 mg of bones, teeth or dry tissues were weighed, placed into sterile aluminum foil, and pulverized in liquid nitrogen. The powdered sample was then digested at 55 °C overnight on a shaker, with 500 μ L digestion buffer (pH 8) containing 0.5% sodium dodecyl sulfate, 0.5 M EDTA, and 100 μ g/mL proteinase K. After 24 h, the digest was purified with silica columns from Qiaquick PCR purification kit (Qiagen). A negative control was included throughout the extraction phase to monitor contamination.

Amplification and Sequencing of mtDNA

To ensure sequence accuracy and authenticity, each mtDNA fragment was amplified and sequenced for every specimen at least twice. Twelve sets of cytoplasmic mitochondrial (Cymt)-specific primers

(Kim *et al.* 2006) designed previously (Driscoll *et al.* 2009; Mondol *et al.* 2009) based on the 4078 bp voucher tiger mtDNA haplotypes (Luo *et al.* 2004) were used to amplify fragments below 250 bp each (Supplementary Table S2 online). These primer sets produced a concatenated mtDNA haplotype of 1750 bp encompassing genetic variations across tiger subspecies in *ND5*, *ND6*, *CytB*, control region, *ND2*, and *COI*.

PCR amplifications were optimized to amplify difficult and degrade DNA, in a 25- μ L reaction system containing 5.0 mM MgCl₂, 1.0 mM dNTPs, 0.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems), 1 \times PCR buffer II, 0.4 μ M each of forward and reverse primer, and 5–7 μ L of extracted DNA. The amplification protocol was: denaturation 10 min at 94 °C, a touchdown cycle of 94 °C for 30 s, annealing at 55 °C for 30 s decreased by 2 °C every 2 cycles until reaching 45 °C, 72 °C for 45 s, then 40 amplification cycles of 95 °C for 30 s, 45 °C for 30 s, and 72 °C for 45 s, followed by a final extension of 10 min at 72 °C. PCR products were incubated with 1.9 units of Exonuclease I (ExoI, GE Healthcare Ltd.) and 0.37 units of Shrimp Alkaline Phosphatase (SAP, GE Healthcare Ltd.) per 10 μ L reaction system at 37 °C for 15 min and 80 °C for 15 min, and subsequently sequenced on an ABI 3730XL sequencing system (Applied Biosystems) as described previously (Luo *et al.* 2004). Sequences were inspected in Sequencher v5.0 (Gene Codes Co.) and compared with the known genetic variable sites from voucher tiger subspecies (Luo *et al.* 2004).

Phylogenetic Analysis

Sequenced mtDNA segments from historic Sundaic tiger specimens were concatenated and assembled into haplotypes based on extended 4078 bp mtDNA sequences (Luo *et al.* 2004) with missing data (due to sequence omission in several samples) coded as “N.” These haplotypes were analyzed jointly with 25 voucher tiger haplotypes of 4078 bp each from all 6 living subspecies (Luo *et al.* 2004) and 1 major haplotype of 1198 bp from historic samples of *P. t. virgata* (Driscoll *et al.* 2009). Two *Panthera* species that are the most closely related to *P. tigris*, or the leopard *P. pardus* (Genbank EF551002) and snow leopard *P. uncia* (Genbank NC_010638), and the sister taxa to *Panthera* spp., the clouded leopard *Neofelis nebulosa* (Genbank DQ257669) were used as outgroups based on 4078 bp homologous mtDNA sequences compiled from complete mtDNA sequences.

Phylogenetic relationships among mtDNA haplotypes were assessed using maximum parsimony (MP), neighbor-joining (NJ), maximum likelihood (ML), and Bayesian approaches. An MP analysis using a heuristic search with random additions of taxa and tree-bisection-reconnection branch swapping and an NJ analysis with NJ trees constructed from Kimura 2-parameter distances followed by tree-bisection-reconnection branch swapping, were conducted in PAUP v4.0b10 (Swofford 2001). Bayesian Information Criterion (BIC) implemented in jModelTest v2.1.4 (Posada 2008) was used to select the best-fit substitution model for ML analysis and Bayesian inference. The HKY85+G model [Base = (0.3246 0.2915 0.1336), Nst = 2, TRatio = 27.6112, Rates = gamma, Shape = 0.1670, Ncat = 8, Pinvar = 0] was selected as the optimal model. Using model parameters, the ML analysis was implemented in PAUP* v4.0b10 and the Bayesian inferences were executed in MrBayes v3.2.0 (Ronquist and Huelsenbeck 2003). Bayesian analysis was performed with 2 simultaneous, independent Markov chain Monte Carlo (MCMC) runs starting from different random trees, each with 3 heated chains and 1 cold chain for 2 000 000 generations. Trees were sampled every 100 generations and the first 25% generations were discarded as burn-in. The reliability of tree topologies was assessed by 2000 bootstrap iterations for the MP and NJ approaches and 100 for ML. Phylogenetic trees were displayed by FigTree v1.3.1. In addition, a statistical parsimony network of mtDNA haplotypes was built from all 9 voucher tiger subspecies using TCS1.1.3 (Clement *et al.* 2000) to examine phylogenetic relationships between voucher tiger subspecies mtDNA haplotypes (Luo *et al.* 2004; Driscoll *et al.* 2009) and the ones carried by the extinct Javan and Bali tigers reported from this study.

Population Genetic Analysis

The extent of population genetic differentiations among putative tiger subspecies and pair-wise differences were assessed by F_{ST} values (with Kimura 2-parameter distance) using analysis of molecular variance (AMOVA) implemented in Arlequin v3.5 (Excoffier *et al.* 2005). Statistical significance was tested using 1000 permutations. Population genetic data was derived from all voucher specimens including mtDNA haplotypes and numbers of individuals carrying the haplotype in each subspecies. Haplotypes of 4078 bp ($N = 105$, including 13 Amur, 7 South China, 32 Indochinese, 22 Malayan, 16 Sumatran, and 15 Bengal tigers) were used for voucher specimens derived from modern samples (Luo *et al.* 2004), 1198 bp ($n = 17$) for Caspian tigers (Driscoll *et al.* 2009), and 1750 bp were compiled for Javan ($N = 9$) and Bali ($N = 2$) tiger specimens from this study.

The time to the most recent common ancestor (TMRCA) for the mtDNA haplotypes among subspecies was estimated in BEAST v1.6.2 (Drummond and Rambaut 2007) with an extended Bayesian skyline plot coalescence model. Speciation times estimated (Johnson *et al.* 2006) for *N. nebulosa* vs. *Panthera* spp. (6.37 MYA, 95% CI = 4.47–9.32 MYA), and for *P. pardus* vs. *P. uncia*/*P. tigris* (3.72 MYA, 95% CI = 2.44–5.79 MYA) were used as calibrations. Nucleotide substitution and site heterogeneity models were estimated from jModelTest v2.1.4 (Posada 2008). The HKY85+G (with rates across sites assumed to follow a continuous gamma distribution) model was selected as the optimal model. Linear model type was set with mitochondrial ploidy and randomly generated starting trees. A strict-clock model and an uncorrelated lognormal relaxed clock model were implemented, and the latter was selected as the best-fit model based on Bayes Factor provided by Tracer v1.5 (Rambaut and Drummond 2009). The MCMC analyses were performed with 4 independent runs simultaneous for 100 000 000 generations each, samples were drawn every 1000 steps and a burn-in of the first 25% was discarded. Validity of estimates was inspected in Tracer v1.6 (Rambaut and Drummond 2009). All runs produced the same parameter distributions, thus the 4 independent runs were combined using LogCombiner v1.8.0. The values of TMRCA and the consensus tree were generated in TreeAnnotator v1.8.0.

To detect past population dynamics of tigers from the Sundaic region (*P. t. sumatrae*, *P. t. sondaica*, and *P. t. balica*), a Bayesian skyline plot was constructed based on mtDNA sequences from 39 samples using BEAST v1.6.2 (Drummond and Rambaut 2007). Site model parameters were estimated in jModelTest v2.1.4 (Posada 2008) and the number of groups was set to 2 based on pairwise log Bayes factor comparison in Tracer v1.5 (Rambaut and Drummond 2009). The piecewise-linear skyline model, randomly generated starting trees, and a strict-clock model were set. Four independent MCMC chains were run for 100 000 000 generations each and parameters sampled every 1000 steps; the first 25% was discarded as burn-in. Output examination and Bayesian Skyline reconstruction were conducted in Tracer v1.6.

Results

DNA was extracted and amplified from 23 (of 33) historic tiger specimens from the Sunda Islands (Table 1), 11 of which were voucher specimens of *P. t. sondaica* (from Java) and *P. t. balica* (from Bali), 6 assigned as *P. t. sondaica* with confidence based on morphometric characters (Yamaguchi *et al.* 2013), and the rest 6 were of unknown geographic records and identified preliminarily as either *P. t. sondaica* or *P. t. sumatrae*. Each specimen was successfully sequenced for at least 8 of the 12 short amplicons that included 6 mtDNA genes (1750 bp) diagnostic of subspecies genetic structure (Luo *et al.* 2004; Luo *et al.* 2008; Driscoll *et al.* 2009). Sequenced fragments were concatenated and aligned with homologous regions from 7 reported voucher tiger subspecies (Luo *et al.* 2004; Driscoll *et al.* 2009). Of the 31 variable sites from mtDNA sequences, 15 synapomorphic characters were diagnostic for subspecies designations in tigers, including 2 sites diagnostic for *P. t. altaica*/*P. t. virgata*, 1 for *P. t. altaica* only, 5 for *P. t. amoyensis*, 2 for *P. t. corbetti*, 2 for *P. t. tigris*, and 2 for *P. t. sumatrae* / *P. t. sondaica* / *P. t. balica*, and 1 for *P. t. sondaica* only. Thirteen variable sites specify polymorphic signature markers, limited to, and diagnostic particular for subspecies (Table 2). For example, there are no synapomorphic sites in *P. t. jacksoni*, whereas 6 polymorphic signature nucleotides are uniquely found in the subspecies.

The 23 tigers from the Sunda Islands carried 6 mtDNA haplotypes (Table 2), 3 of which were new haplotypes (SON, BAL, and SUMx) and 3 identical to voucher Sumatran tiger haplotypes (SUM1/2/5, SUM3, and SUN6/7/8; Luo *et al.* 2004). All known Sumatran tiger haplotypes detected are carried by nonvoucher specimens ($N = 5$), none of which has a definite geographic origin, though identified as related to *P. t. sumatrae* to a certain extent (Table 1). SUMx is 1696 bp and found in 1 specimen (Leiden 4700), whose exact origin in Indonesia is not known. Haplotype SON carries a unique SNP (ND6-14698-G; Table 2), found in 11 *P. t. sondaica* defined by either geographic records from Java ($N = 9$) or morphometric assignments ($N = 2$). Specimens carrying haplotype SON were collected from museums in the Netherlands, China, Russia, and Germany, and represented all voucher specimens of *P. t. sondaica* in our collection ($N = 9$). Haplotype BAL has no specific or novel site and is a single-nucleotide step from SON (ND6-14698-G) and Sumatran tiger haplotype SUM1/2/5 (ND5-13173-A; Table 2). The ND5-13173-A variant shared by BAL and SON distinguishes them from all Sumatran tiger haplotypes (SUM1-8 and SUMx). BAL is common in our collection and shared by 6 individuals (Table 1), including 2 voucher specimens of *P. t. balica* from Bali (Stuttgart 18922 and 18923) that represent all voucher Bali tigers in the collection and 4 assigned *P. t. sondaica* whose exact origin is not known but skull morphometric characters resemble other voucher Javan tiger specimens with high confidence (Yamaguchi *et al.* 2013). All 3 new haplotypes (SON, BAL and SUMx) share the 2 Sumatran tiger diagnostic substitutions, *CytB* 15743-G and ND2-5608-T (Table 2), and differ from most similar Sumatran tiger haplotypes by only 1 or 2 nucleotides.

Geographic distribution of mtDNA haplotypes among voucher tiger specimens with recorded origins from the islands of Sumatra, Java, and Bali indicates a recent common ancestor among these subspecies (Figures 1 and 2). SON and BAL were 2 haplotypes exclusively found in Java and Bali; none of the 8 haplotypes from the living voucher Sumatran tigers (SUM1-8 from Luo *et al.* 2004) existed in Java or Bali. No mtDNA haplotype is shared among voucher Sumatran, Javan and Bali tigers. All 9 voucher Javan tigers carry haplotype SON and both voucher Bali tigers carry BAL.

Phylogenetic analysis of mtDNA haplotypes representing all 9 tiger subspecies using MP, NJ, ML, and Bayesian approaches produced congruent topologies corresponding to major geographic partitions. Tigers from the 3 Sunda Islands clustered into a monophyletic group with 11 haplotypes. The 2 haplotypes (SON and BAL) in voucher *P. t. sondaica* and *P. t. balica* formed a statistically robust subgroup within the Sunda tiger population (Figure 2). Mainland Asian tigers parse into 5 distinct groups corresponding to major phylogeographic clustering and prior subspecies recognition (Luo *et al.* 2004), including the Bengal tiger *P. t. tigris*, South China tiger *P. t. amoyensis*, Malayan tiger *P. t. jacksoni*, Indochinese tiger *P. t. corbetti*, Amur tiger *P. t. altaica* and Caspian tiger *P. t. virgata* differing from *P. t. altaica* by a single nucleotide (Driscoll *et al.* 2009). *P. t. virgata*, *P. t. altaica*, and *P. t. corbetti* formed one larger monophyletic association. *P. t. tigris* on the Indian subcontinent is genetically distinct from other mainland subspecies, corresponding to an early divergence (Mondol *et al.* 2009). The phylogenetic placement of *P. t. amoyensis* is considered basal in modern tiger evolution (Herrington 1987; Driscoll *et al.* 2009) and is consistent with MP and NJ approaches here

(bootstrap values = 59 and 62, respectively, Figure 2A), although statistical support from ML is not high (bootstrap value = 46) and Bayesian phylogenetic analysis indicates a contemporaneous radiation of major modern tiger mtDNA lineages (Supplementary Figures S1 and S2 online).

We quantified the extent of population differentiation in modern tigers including all extant and extinct subspecies on the basis of AMOVA and mtDNA haplotypes (Table 3). All pair-wise population genetic distinctions as evaluated by F_{ST} were significant ($P < 0.05$). The average F_{ST} was high at 0.916, in support of the 9 subspecies classification.

The estimated coalescence time of mtDNA haplotypes for all tiger subspecies (Supplementary Figure S2 online) using *Panthera* spp. divergence times as calibration (Johnson *et al.* 2006) is 94 500 years (95% CI = 47 900–158 000 years), highly consistent with a previous estimation of 108 000 years (95% CI = 59 000–157 000 years, Luo *et al.* 2004). The recent coalescence in modern tigers, as compared to some other *Panthera* species (e.g., 470 000–825 000 years in the leopard and 280 000–510 000 years in the jaguar; Eizirik *et al.* 2001; Uphyrkina *et al.* 2001), is consistent with a late Pleistocene bottleneck in tigers (Luo *et al.* 2004). Bayesian skyline plot of mtDNA sequences from Sumatran, Javan, and Bali tigers indicated an overall stable and large effective population size of tigers in the Sunda region, with a moderate increase since the last glacial period (Figure 3).

Discussion

Taxonomic Status of Javan and Bali Tigers

Our results provide consistent molecular genetic evidence that tigers on Bali, Java, and Sumatra recently derived from a common matrilineal genetic lineage (Figures 1 and 2; Tables 2 and 3; Supplementary Figure S1 online). This mtDNA similarity needs to be validated by nuclear genetic evidence yet the close association among the 3 subspecies is supported by the clustering of all mtDNA haplotypes from the 3 Sunda Islands leading to a strongly supported monophyletic clade distinct from other mainland subspecies; the existence of a recent ancestral Sunda tiger lineage that later evolved into present populations on Java, Bali, and Sumatra.

Previous paleontological and morphological studies have suggested that the Sumatran tiger is a hybrid of mainland tigers and the Javan tiger (Mazak 2010); however, this conclusion is not supported by maternal mtDNA or bi-parental nuclear microsatellite markers (Luo *et al.* 2004). In 1969, Hemmer suggested a differentiation between Sumatran and Javan/Bali tigers based on skin and skull morphology (Hemmer 1969), whereas Javan and Bali tigers are broadly similar except for overall smaller size in the latter (Mazak and Groves 2006; Mazak 2010; Yamaguchi *et al.* 2013). J. H. Mazák and Groves also supported separation of the Sumatran tiger from the Javan and Bali tigers based on craniometrical data collected by the late V. Mazák (Mazak and Groves 2006; Mazak 2010) and several diagnostic cranial characteristics have been proposed (Yamaguchi *et al.* 2013). Our molecular phylogenetic and phylogeographic analysis supports geographic subdivision within the Sunda tiger group, with haplotypes SON and BAL exclusively found from Java and Bali respectively (Figure 1). The genetic differentiation between Sumatran, Javan and Bali tigers is also significant (Table 3), indicating a restriction or lack of matrilineal gene flow among the three islands. Whether increased sampling of voucher tiger specimens or nuclear genomic data would collapse such a

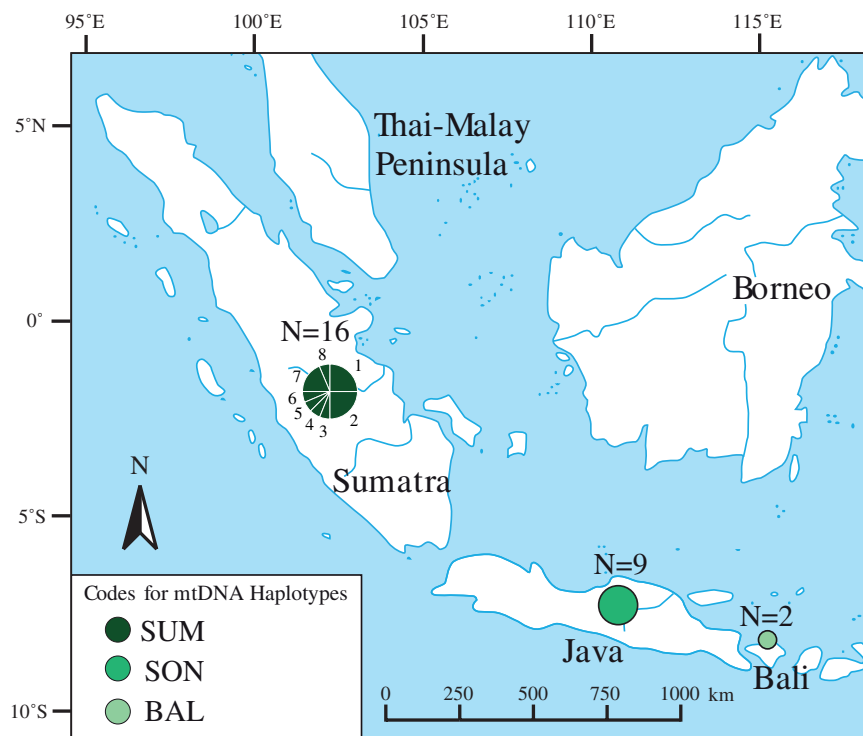


Figure 1. Geographic distribution of mtDNA haplotypes among tigers from the islands of Sumatra, Java, and Bali. Only tiger specimens with confirmed geographic origins from Java ($N=9$) and Bali ($N=2$) are used (Table 1). Haplotypes are color-coded. SUM includes 8 mtDNA haplotypes represented by voucher Sumatran tigers ($N=16$) (Luo *et al.* 2004). SON and BAL are 2 haplotypes exclusively found in Java and Bali different from the most closely related SUM haplotype by 1 or 2 bp only (Table 2). No haplotype is shared among voucher Sumatran, Javan and Bali tigers. The size of a circle corresponds to the number of specimens from the island and sectors of the pie chart are proportional to haplotype frequencies.

distinction requires further studies. An enhanced sampling coverage from the islands of Java, Bali, Sumatra, and even Borneo and Palawan that were likely colonized by tigers prehistorically (Piper *et al.* 2008), may help further elucidate the issue. Nevertheless, the similarity among the Sunda tiger mtDNA haplotypes (1–2 nucleotide difference among Sumatran, Javan and Bali tigers) suggests common origin and rapid divergence of island subspecies, and may reflect that the somewhat distinctive morphological features in each subspecies have evolved rapidly after each island was colonized.

The time to the most recent common ancestor of modern tigers has been estimated at 72 000–108 000 years depending on fossil calibration time for tiger-leopard divergence at 2 MYA or 3 MYA (Luo *et al.* 2004), or at 94 500 years (95% CI = 47 900–158 000 years; Supplementary Figure S2 online) here when using *Panthera* spp. divergence time from Felidae phylogeny (Johnson *et al.* 2006) as calibration. All estimates consistently put the coalescence time of modern tiger lineages within the last 100 000 years. Mitochondrial DNA haplotypes from tigers from Java and Bali fall into the Sunda tiger clade that belongs to modern tigers. Therefore, these genetic data do not support the hypothesis that the modern Javan tiger is an autochthonous descendant of a prehistoric tiger population, whose fossils were found in Java and dated to 1.3–2.1 MYA (Hemmer 1971; Hemmer 1987; Kitchener and Yamaguchi 2010). Instead, early to middle Pleistocene tiger populations in the Sunda Islands may have been eliminated because of drastic biogeographical events associated with glacial–interglacial oscillations and/or the Toba volcano super-eruption c. 73 500 years ago (Rampino and Self 1992; Williams *et al.* 2009). Recolonization of modern tigers of the Sunda

Islands after the Late Pleistocene demographic bottleneck was possible when the islands were connected intermittently during periods of glacial cycles until the end of LGM 20 000 years ago (Bassett *et al.* 2005). However, to investigate these hypotheses scientifically, we need further and more detailed evidence that may become available in the future.

Overview of Modern Tiger Evolutionary History

We are now able to construct, for the first time, the intraspecific phylogeny for the tiger based on all the 9 recognized subspecies. Interpreting the layered cladogenic effects of variance and dispersal illustrated in Figure 2 (also see Table 3; Supplementary Figures S1 and S2 online) presents a plausible solution to longstanding uncertainty regarding the tiger radiation throughout mainland Asia and the Sunda Islands. The position of *P. t. amoyensis* and its relation to *P. t. sumatrae*/*P. t. sondaica*/*P. t. balica* in the phylogenetic tree suggests a once widespread tiger population from China to the Sunda Shelf that became isolated, likely by rising sea levels during interglacial periods (Mazak 1968; Kitchener and Yamaguchi 2010). A second wave of tiger expansion and divergence (*P. t. tigris*, *P. t. corbetti*, *P. t. jacksoni*, *P. t. altaica*, and *P. t. virgata*) replaced much of the range of *P. t. amoyensis* on the mainland and evolved into modern populations in Indochina, the Indian Subcontinent, the Caucasus, and Russian Far East, where tiger fossils are only found from the Holocene (Mazak 1968; Heptner and Sludskii 1972; Kitchener and Dugmore 2000; Kitchener and Yamaguchi 2010). Demographic history reconstruction of tigers from the islands of Sumatra, Bali, and Java

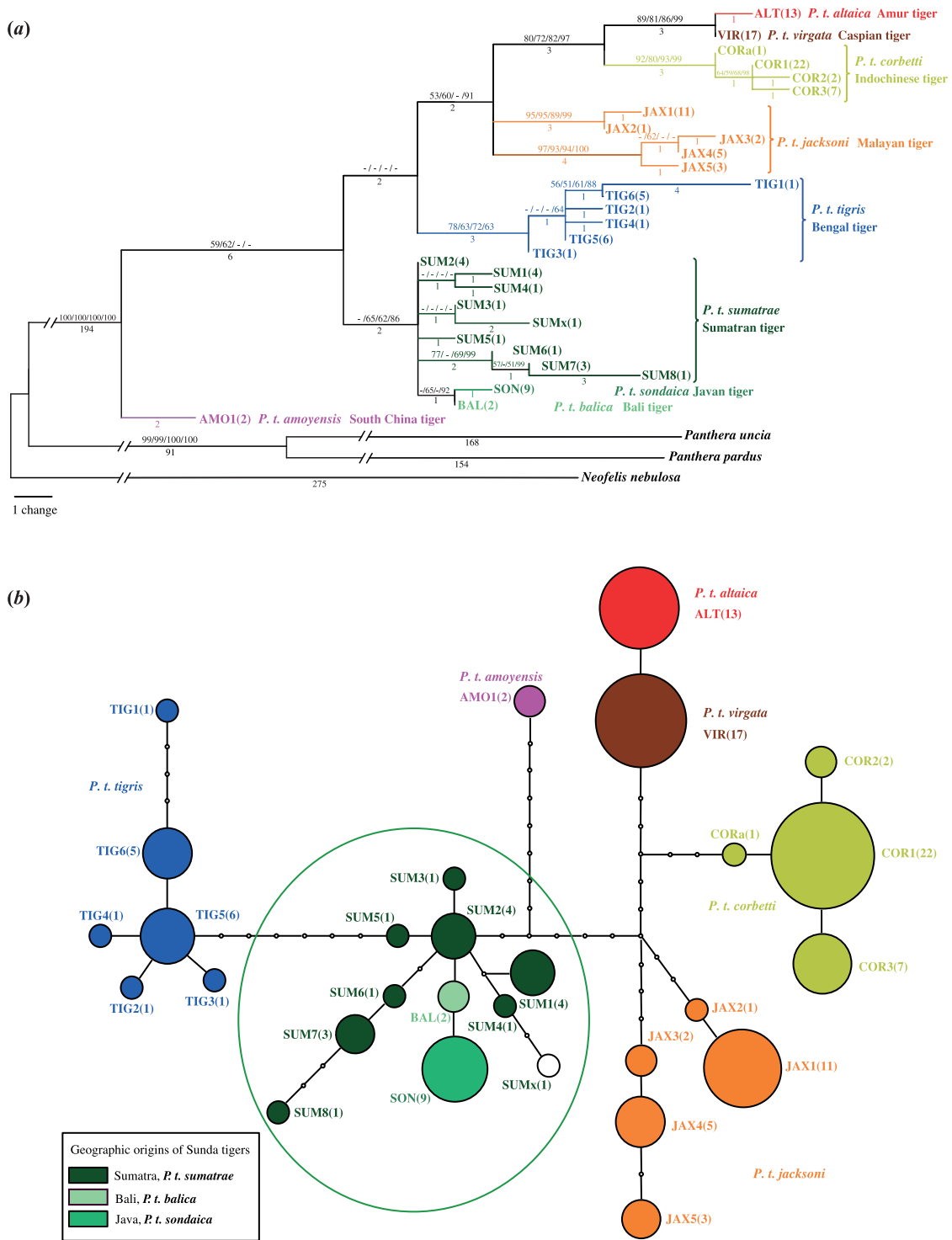


Figure 2. Phylogeny of *Panthera tigris* mtDNA sequences. (a) Phylogenetic relationships among tiger mtDNA haplotypes based on 4078bp of concatenated sequences from all 9 subspecies rooted with *P. uncia*, *P. pardus*, and *N. nebulosa*. Fragments obtained from Sundaic historic tiger specimens are concatenated into mtDNA haplotypes of 1750 bp in SON and BAL, and 1629 bp in SUMx, respectively, and analyzed jointly with other previously published voucher haplotypes (Luo et al. 2004; Driscoll et al. 2009). Haplotype designations are color-coded by subspecies. Eleven closely related haplotypes from *P. t. sumatrae*, *P. t. sondaica*, and *P. t. balica* form a monophyletic group with strong statistical support. Phylogenetic trees derived from maximum parsimony (MP), neighbor-joining (NJ), maximum likelihood (ML), and Bayesian analyses have similar topologies and only the MP tree is shown. Numbers above branches represent bootstrap support in percent using the MP (from 2000 replicates), NJ (from 2000 replicates), and ML (from 100 replicates) methods, followed by posterior probabilities using Bayesian analyses (only those over 50% are indicated). Numbers below branches show the number of changes. (b) Statistical parsimony network of 29 mtDNA haplotypes (4078 bp) as represented by circles from all 9 tiger subspecies. Circle size is proportional to the number of samples sharing the haplotype. BAL is shared by both voucher tiger specimens from Bali, and is one step away from SUM2. SON is 2 nucleotides different from SUM2 and found in tigers from Java only.

Table 3. Pair-wise genetic differentiations (F_{ST})^a among tiger subspecies^b based on AMOVA with mtDNA data

	ALT (N = 13)	VIR (N = 17)	AMO (N = 7)	COR (N = 32)	JAX (N = 22)	SUM (N = 16)	SON (N = 9)	BAL (N = 2)	TIG (N = 15)
ALT	—								
VIR	1.000	—							
AMO	1.000	1.000	—						
COR	0.954	1.000	0.974	—					
JAX	0.742	0.842	0.757	0.797	—				
SUM	0.891	0.956	0.831	0.910	0.668	—			
SON	1.000	1.000	1.000	0.994	0.737	0.817	—		
BAL	1.000	1.000	1.000	0.991	0.610	0.581	1.000	—	
TIG	0.942	0.963	0.932	0.940	0.691	0.786	0.935	0.891	—

^aPopulation pairwise F_{ST} is calculated using the combined 4078 bp of mtDNA haplotypes (except for 1750 bp of SON and BAL and 1198 bp of VIR) and Kimura two parameter. All pair-wise comparisons are significant ($P < 0.05$). Sample size for all tiger subspecies voucher samples is 133 (Luo *et al.* 2004).

^bAbbreviations for subspecies: ALT, Amur tiger *P. t. altaica*; VIR, Caspian tiger *P. t. virgata*; AMO, South China tiger *P. t. amoyensis*; COR, Indochinese tiger *P. t. corbetti*; JAX, Malayan tiger *P. t. jacksoni*; SUM, Sumatran tiger *P. t. sumatrae*; SON, Javan tiger *P. t. sondaica*; BAL, Bali tiger *P. t. balica*; TIG, Bengal tiger *P. t. tigris*.

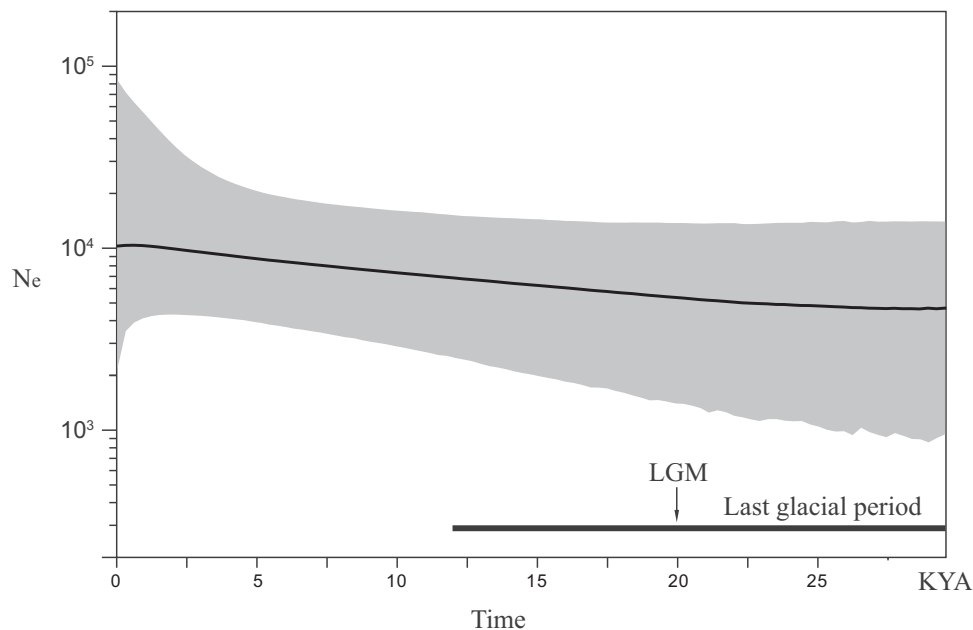


Figure 3. Bayesian skyline plot of mtDNA sequences of tigers from the islands of Sumatra, Java, and Bali. The x-axis is time in KYA (thousand years ago). The last glacial period (c. 110 000–12 000 years ago) and last glacial maximum (LGM, c. 20 000 years ago) are marked. The y-axis represents effective population size N_e , assuming a generation time of 5 years for the tiger (Smith and McDougal 1991). The solid black line shows estimate of the posterior median and the shading denotes 95% highest posterior density limits.

(Figure 3) indicates modest and recent population expansion throughout the last glacial period (c. 110 000–12 000 years ago) in the region. Inference with the Pair-wise Sequentially Markovian Coalescent (PSMC) model of tiger demographic history based on genome-wide data from an Amur tiger also indicates a Holocene population expansion as ice sheets retreated and suitable habitats containing ungulate prey returned (Cho *et al.* 2013). Subspecies differentiation in tigers is likely a result of geographic isolation, genetic drift, and local adaptation associated with repeated restriction and expansion of habitats in the last 100 000 years (Luo *et al.* 2004; Luo *et al.* 2010b).

Conservation Implications

Javan and Bali tigers became extinct because of poaching and the loss of habitat and prey (Chundawat *et al.* 2011). The last record

of a Bali tiger was in the 1930s (Seidensticker 1987); the last reliable sighting of a Javan tiger occurred in 1976 (Seidensticker 1987); and in Sumatra, timber production, forest conversion to agriculture and settlements, poaching, and the trade in tiger parts continue to threaten tiger survival. As the Sumatran tiger is the last living representative of the Sunda tigers, conservation must preserve and increase the 400 wild tigers that remain here (Dinerstein *et al.* 2007; Chundawat *et al.* 2011). Our results based on mtDNA suggest that the 3 Sundaic tiger subspecies are phylogenetically more closely related to each other than was previously suggested. This close genetic relationship may raise the prospect of a managed restoration of suitable and prey-enriched habitats in Bali and Java with tigers sourced from wild or captive populations of Sumatran tiger, as has been proposed to restore the Caspian tiger (Driscoll *et al.* 2009; Driscoll *et al.* 2011).

Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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