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Report

Molecular Evidence for Species-Level Distinctions in Clouded Leopards

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Summary

Among the 37 living species of Felidae, the clouded leopard (*Neofelis nebulosa*) is generally classified as a monotypic genus basal to the *Panthera* lineage of great cats [1–5]. This secretive, mid-sized (16–23 kg) carnivore, now severely endangered, is traditionally subdivided into four southeast Asian subspecies (Figure 1A) [4–8]. We used molecular genetic methods to re-evaluate subspecies partitions and to quantify patterns of population genetic variation among 109 clouded leopards of known geographic origin (Figure 1A, Tables S1 and S2 in the Supplemental Data available online). We found strong phylogeographic monophyly and large genetic distances between *N. n. nebulosa* (mainland) and *N. n. diardi* (Borneo; n = 3 individuals) with mtDNA (771 bp), nuclear DNA (3100 bp), and 51 microsatellite loci. Thirty-six fixed mitochondrial and nuclear nucleotide differences and 20 microsatellite loci with nonoverlapping allele-size ranges distinguished *N. n. nebulosa* from *N. n. diardi*. Along with fixed subspecies-specific chromosomal differences, this degree of differentiation is equivalent to, or greater than, comparable measures among five recognized *Panthera* species (lion, tiger, leopard, jaguar, and snow leopard). These distinctions increase the urgency of clouded leopard conservation efforts, and if affirmed by morphological analysis and wider sampling of *N. n. diardi* in Borneo and Sumatra, would support reclassification of *N. n. diardi* as a new species (*Neofelis diardi*).

Introduction

Patterns of phylogeographic variation and the validity of current taxonomic delineations were evaluated with a variety of molecular genetic markers that, together, provide a rigorous assessment of distinctiveness among groups of clouded leopards. The same measures were assessed among the five well-accepted species of the *Panthera* lineage to provide an evolutionary context and a direct comparison.

Mitochondrial DNA

MtDNA sequence variation was assessed from four gene segments, *ATP-8* (139 bp), control region (190 bp), *Cyt-b* (219 bp), and *ND5* (223 bp), which were analyzed both separately and together (Figures 1B and 1C; Figures S1 and S2). DNA sequences were obtained for all four subspecies of clouded leopard for only the 139 bp of the *ATP-8* gene fragment (Figure 1B; Figures S1 and S2). Among these sequences, there were 12–14 variable sites that distinguished *N. n. diardi* from the other three subspecies (Table S3). This genetic distance is similar to the 5–16 nucleotide differences among *Panthera* species. Of 14 variable *ATP*-8 sites among clouded leopard subspecies, five had nucleotides found only in *N. n.* diardi (Borneo) and not in other clouded leopard subspecies or *Panthera* species (Figures S1 and S2). There were six *ATP*-8 haplotypes (Figure 1B; Figures S1 and S2) among 67 clouded leopards (including four museum samples). *N. n. diardi* from Borneo (n = 3) had two haplotypes (DIA-1, DIA-2), *N. n. macrosceloides*

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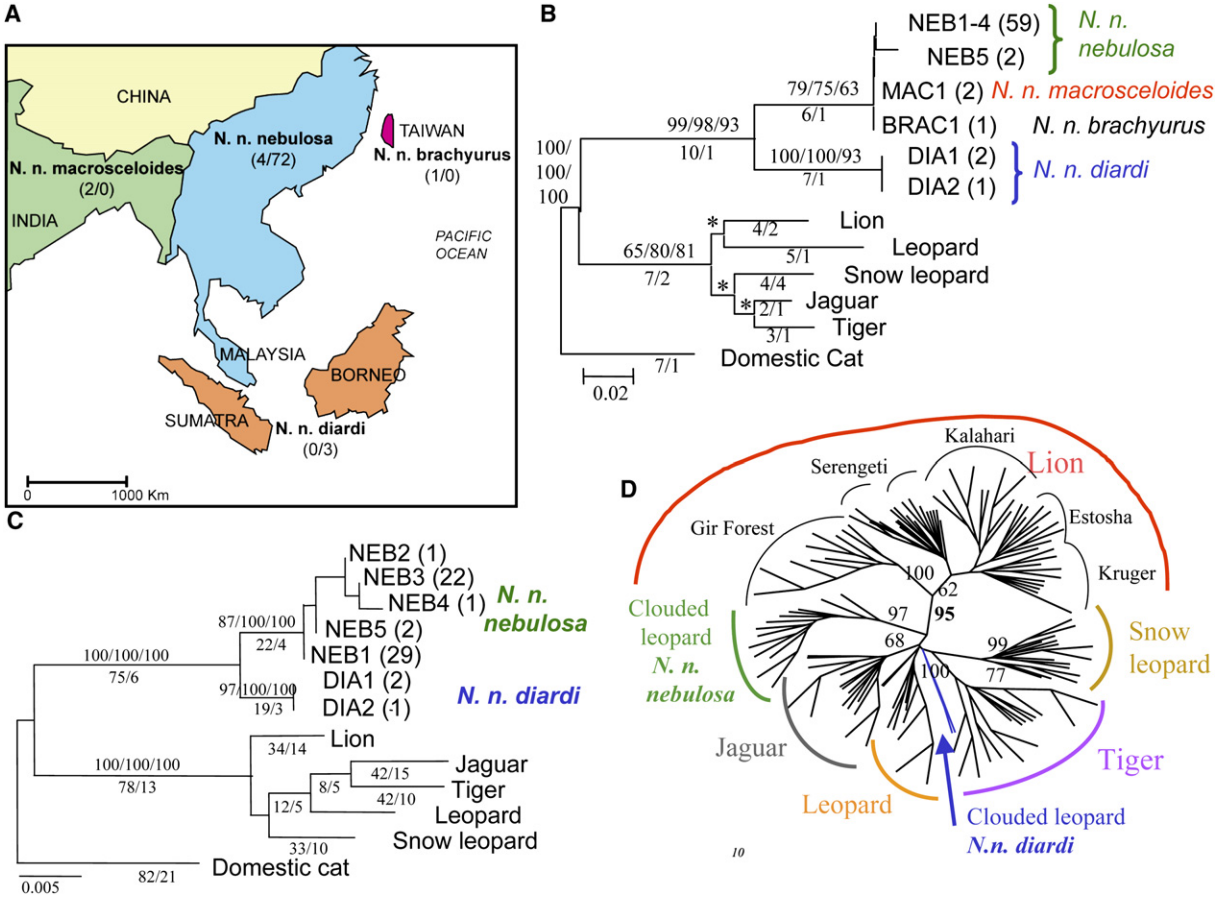

Figure 1. Geographic and Phylogenetic Depiction of Individuals Used in This Study

(A) Geographic range of the four currently recognized clouded leopard subspecies based upon historical descriptions. The numbers depicted before the slash mark indicate the number of samples that amplified robustly and were used in the study. Those numbers depicted after the slash marks indicate the total number of samples that were collected for analysis. Ancient, or museum, samples are listed separately from modern samples in Tables S1 and S2. The three available *N. n. diardi* samples were born in the wild in Borneo (Table S1).

(B) Phylogenetic relationships among haplotypes of *Neofelis* and *Panthera* genera were rooted with the domestic cat and based on analysis of the *ATP-8* (139 bp) mtDNA gene segment. An asterisk denotes less than 60% bootstrap support.

(C) Combined mtDNA and nuclear gene segments (3.9 Kb). Depicted phylogenetic trees were constructed by minimum evolution (ME) with neighbor-joining (NJ) algorithm and Kimura 2 distances. Maximum parsimony (MP) and maximum likelihood (ML) phylogenetic trees were also constructed and showed similar topology. Bootstrap values from 100 iterations are listed above the lines at major nodes for each of the three methods (NJ/MP/ML). Below the branches is the number of steps/number of homoplasies. To the right of each haplotype (in parentheses) is the number of clouded leopard individuals with this haplotype (for mtDNA) or genotype (for nuclear genes).

(D) Unrooted neighbor-joining phylogram constructed by Dps (Proportions of Shared Alleles) based upon each individual's composite microsatellite genotype. Bootstrap support (100 iterations) is indicated.

(n = 2) had one haplotype (MAC-1), and *N. n. brachyurus* (n = 1) had haplotype BRAC-1. Two haplotypes (NEB-3, NEB-5) were found in *N. n. nebulosa* (n = 61).

In a phylogenetic analysis of the 139 bp of ATP-8, the three mainland subspecies fell into a monophyletic cluster distinctive of *N. n. diardi* with bootstrap support (BS) ranging from 63%–100% for three phylogenetic analytical methods (Figure 1B). *N. n. diardi* samples (haplotypes DIA-1 and DIA-2) formed a distinctive group with high BS for all three methods (100/100/93). The three other *N. nebulosa* subspecies formed an unresolved group (BS of 79/75/63). Similarly relationships among *Panthera* species were unresolved (Figure 1B).

When the four mtDNA gene segments (771 bp) were analyzed as a whole, clouded leopards were monophyletic relative to the other *Panthera* species (Figure S3). Thirty-five *N. n. nebulosa* had five unique haplotypes, whereas the three *N. n. diardi* carried two distinctive haplotypes. There were 27–31 nucleotide differences separating *N. n. nebulosa* individuals from *N. n. diardi* (Bornean), as compared with 42–60 nucleotide differences separating pairs of *Panthera* species (Table S3B).

Nuclear DNA Sequence

Nuclear gene sequences (nDNA) of *ATP-7A* (635 bp), *BGN* (610 bp), *HK1* (338 bp), *IDS* (573 bp), and *PLP* (932 bp) were each analyzed individually, then together as a nDNA data set, and finally combined with the mtDNA sequences (Figure S4). Using the concatenated nDNA genes, we found 11 sites that defined one unique combined sequence or "haplotype" in the three members of *N. n. diardi* (DIA-1) (Figure S2, Table S3C) and one haplotype (NEB-3) unique to all *N. n. nebulosa* samples (Figure 1B). Among the *Panthera* species there

Table 1. Distance Matrix Listing the Number of Nucleotide Differences for All Gene Segments Combined

	DIA-1	DIA-2	NEB-1	NEB-2	NEB-3	NEB-4	NEB-5	Lion	Jaguar	Leopard	Tiger	Snow leopard	Domestic cat
DIA-1	*												
DIA-2	1	*											
NEB-1	38	38	*										
NEB-2	36	37	1	*									
NEB-3	40	41	1	2	*								
NEB-4	39	39	2	3	1	*							
NEB-5	39	39	1	2	2	3	*						
Lion	140	140	130	122	133	127	133	*					
Jaguar	192	193	187	178	191	187	190	72	*				
Leopard	146	147	148	141	151	145	150	56	68	*			
Tiger	190	192	184	176	188	186	189	59	72	64	*		
Snow leopard	141	142	135	126	140	134	139	59	70	61	57	*	
Domestic cat	175	176	176	161	178	164	178	138	190	134	193	134	*

For each clouded leopard individual, the haplotype listed represents a haplotype shared with other individuals for that same gene segment or segments (Figure S5–S8). DIA, *N. n. diardi*; NEB, *N. n. nebulosa*.

were 7–17 nucleotide differences in nuclear genes segments (Table S3C).

With combined analyses of concatenated mtDNA and nDNA sequences (Figure 1C; Table 1), 36-41 nucleotides separated individuals of *N. n. diardi* from those of *N. n. nebulosa*; in comparison, there were 56–72 nucleotide differences separating *Panthera* species. In the combined 3,859 bp sequence, *N. n. diardi* (Bornean; DIA-1, DIA-2) had 36–41 fixed differences relative to *N. n. nebulosa* (Table 1); 21 of these variable nucleotide differences were also found in at least one *Panthera* species. The remaining 20 differences were unique to *N. n. diardi* and were not shared with *Panthera* species (Figures S1 and S2). Complete sequence data from all genes (771 bp mtDNA and 3088 bp nDNA) were available for only *N. n. nebulosa* and *N. n. diardi* because *N. n. macrosceloides* and *N. n. brachyurus* were represented by museum samples only (Table S2). *N. n. nebulosa* and *N. n. diardi* were reciprocally monophyletic with high bootstrap support (97/100/100 and 87/100/100, respectively) (Figure 1C). There were 41 steps between the subspecies, including seven homoplasies.

Microsatellites

Genetic variation was estimated with 51 felid microsatellites [9] (Table S4) for clouded leopards and five *Panthera* species, including six diverse lion populations (Table 2). Observed heterozygosity for *Panthera* species was Ca 0.5, except in lions, where Ho was 0.389 ± 0.009, ranging from 0.090 ± 0.013 in inbred Gir Forest lions to 0.504 ± 0.022 in the outbred Serengeti National Park lions. Observed heterozygosity across all clouded leopards was 0.471 ± 0.019 (0.482 ± 0.021 in *N. n. nebulosa* and 0.441 ± 0.050 in *N. n. diardi*). Average microsatellite variance ranged from 2.43 to 6.55 within *Panthera* species but was almost double that in clouded leopards (11.38) (Table 2), indicating a large accumulation of mutational variation and suggesting the passage of a relatively long (near species-level) time interval [10, 11].

We evaluated five microsatellite genetic-distance estimators to reveal species and population distinctions among clouded leopards and *Panthera* species (Figure 1D, Figures S5–S8). Each *Panthera* species defined a monophyletic group (see Dps-based phylogram, Figure 1D), with bootstrap support from 68%–99%, and among the lions internal population structure corresponded to subspecies or geographic designations. *N. n. nebulosa* individuals formed a monophyletic group (97% BS) distinct from the two *N. n. diardi*, which formed a highly monophyletic group. Other measures of microsatellite genetic distance [Dkf, Fst, Gst, and $(\delta\mu)^2$] similarly distinguished *N. n. nebulosa* and *N. n. diardi* (Figures S5–S8).

In a comparison of the distribution of microsatellite allele sizes between clouded leopard subspecies (Figure S9), allele sizes for 20 of 51 loci did not overlap between *N. n. nebulosa* and *N. n. diardi* (Fca44, 80, 81, 82, 90, 94, 100, 105, 107, 132, 144, 176, 212, 225, 249, 261, 275, 290, 304, and 310). This is twice the number of nonoverlapping alleles observed between any pair of *Panthera* species (Figure S9), which had from four (between lion and leopard) to nine (between snow leopard and tiger and between tiger and lion) non-size-overlapping loci. For six loci (Fca80, 82, 100, 107, 144, and 275), the allele size gap between *Neofelis* subspecies exceeded 14 bp (Figure S9). Between pairs of *Panthera* species, the maximum gap in allele size ranged from 30 bp between lions and leopards (FCA117) to 10 bp between leopards and jaguars.

Clouded leopard microsatellite data (for the individuals in Figure 1D) were analyzed for evidence of population genetic structure/partitions with ARARst [12]. A two-group scenario partitioning *N. n. diardi* from *N. n. nebulosa* had the highest Rst value (0.60) relative to other scenarios with further partitions and showed significant population genetic differentiation ($p < 0.001$). A Bayesian algorithm as implemented in STRUCTURE [13] also provided the strongest support for only two partitions ($K = 2$, $Pr(K) = 0.644$). In this scenario, all *N. n. nebulosa* and *N. n. diardi* individuals were assigned to two unique clusters with high probability ($q > 0.90$), indicative of low levels of gene flow. We estimated *N. n. nebulosa* diverged from *N. n. diardi* 1.41 million years ago (MYA) (95% CI of 0.93–2.0 MYA) by using a calibration of 6.37 MYA for the divergence of clouded leopards from the *Panthera* lineage based upon a comprehensive analysis of nearly 20 kb of nuclear gene sequence and multiple fossil dates [1].

Cytogenetic Variation

Most felid species, as well as many canid, mustelid, and hyena species, have two distinct acrocentric

Table 2. Estimates of Genetic Variation among 51 Microsatellite Loci in Clouded Leopard Subspecies Compared with Species and Subspecies of *Panthera* and the Domestic Cat

Species or Population	Individual	Loci Typed	Observed heterozygosity ± SD	Expected heterozygosity ± SD	Average Number of Alleles per Locus	Average Microsatellite Variance	Average Allele Size Range (Repeats)/ Locus
Neofelis nebulosa	15	51	0.471 ± 0.0192	0.665 ± 0.0289	5.82 ± 2.26	11.38	9.31
N. n. nebulosa	13	51	0.482 ± 0.0208	0.597 ± 0.0352	4.55 ± 1.99	7.46	6.49
N. n. diardi	2	51	0.441 ± 0.0503	0.487 ± 0.0467	2.10 ± 0.77	2.54	2.16
Panthera onca	15	51	0.624 ± 0.0187	0.717 ± 0.0285	6.33 ± 2.17	5.54	7.69
Panthera pardus	13	49	0.514 ± 0.0236	0.774 ± 0.0252	6.31 ± 2.13	6.55	7.59
Panthera tigris	15	51	0.482 ± 0.0207	0.689 ± 0.0346	5.53 ± 2.06	4.93	6.47
Panthera uncia	15	50	0.515 ± 0.0188	0.577 ± 0.0339	4.20 ± 1.74	2.43	4.30
Panthera leo	60	51	0.394 ± 0.0093	0.560 ± 0.0368	5.29 ± 2.66	4.72	7.23
Gir Forest	10	51	0.090 ± 0.0127	0.096 ± 0.0259	1.31 ± 0.65	0.26	0.55
Ngorongoro Crater	10	51	0.445 ± 0.0224	0.435 ± 0.0355	2.84 ± 1.24	3.02	4.14
Serengeti National Park	10	51	0.504 ± 0.0224	0.524 ± 0.0364	3.61 ± 1.7	4.84	5.49
Kalahari Gemsbok National Park	10	44	0.393 ± 0.0238	0.404 ± 0.0444	2.68 ± 1.38	4.14	3.66
Etosha Park	10	44	0.460 ± 0.0242	0.492 ± 0.0408	3.05 ± 1.43	3.96	3.77
Kruger National Park	10	44	0.499 ± 0.0240	0.480 ± 0.0419	3.32 ± 1.7	4.38	4.80
Felis catus	15	51	0.616 ± 0.0180	0.699 ± 0.0226	6.18 ± 2.46	5.34	7.78

Six previously published [11] lion (*P. leo*) populations were utilized in this analysis. These populations include those of the following locations: Gir Forest, Ngorongoro Crater, Serengeti National Park, Kalahari Gemsbok National Park, Etosha Park, and Kruger National Park. Thirteen domestic-cat genotypes from a previous study [9] and two from this study were combined for analysis for this table.

chromosomes, designated F2 and F3 [14, 15], that are considered part of the ancestral carnivore karyotype [15–17]. A G band analysis of metaphase chromosomes from three *N. n. diardi* and nine *N. n. nebulosa* revealed a dramatic fixed difference between the two subspecies. *N. n. diardi* individuals presented a conventional smaller F3 chromosome, identical to that in other felids. However, nine *N. n. nebulosa* had previously described F3 variants [14, 15] that were larger than F2 variants and that sometimes included an extended chromosomal arm above the centromere (Figure 2). This chromosomal polymorphism is derived from recent additions of constitutive heterochomatin and also occurs in other mammals [16]. Additional insight was derived from chromosome paints of metaphase spreads, with field heterochromatin being visualized with a degenerate oligonucleotide-primed PCR (DOP-PCR) probe from an E group chromosome (R.S., unpublished data). This probe illuminated the homologous E group chromosome of both subspecies (arrows in Figure S10). However, in *N. n. nebulosa* the telomeres of several other chromosomes were also illuminated (Figure S10A), providing additional evidence of an increase in constitutive heterochromatin in *N. n. nebulosa*.

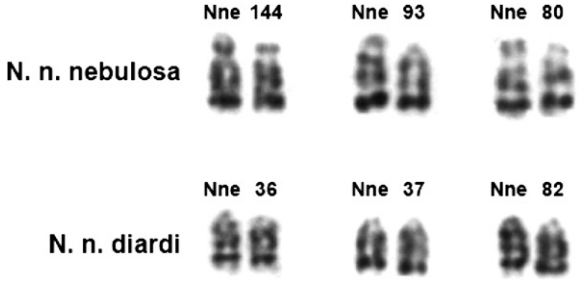

Figure 2. G Band Karyotype of Chromosome F3 from Three *N. n. nebulosa* Metaphase Preparations and Three *N. n. diardi* Preparations

Note that *N. n. diardi* are all acrocentric. The *N. n. nebulosa* F3 chromosomes are larger and polymorphic for the amount of extrachromatin material, either above or below the centromere. The extended F3 chromosome was present in nine *N. n. nebulosa* individuals but not in the three *N. n. diardi* individuals examined.

Discussion

An accurate and formalized taxonomy is critical to the conservation of the clouded leopard. Here we show that Bornean clouded leopards are a distinct population, reproductively isolated from other clouded leopard subspecies and having clear phylogenetic discontinuities. We estimate that Bornean clouded leopards diverged from mainland populations during the Pleistocene, when recurring episodes of global cooling and warming created opportunities for population isolation. The Sunda Shelf, between the Indonesian archipelago and Vietnam, was repeatedly exposed and then covered by changing sea levels [18]. However, even when the archipelago was connected to the mainland [19, 20], ancient river systems may have continued to isolate modern Borneo [20, 21]. Although captive breeding between Bornean and mainland clouded leopards has not been documented to our knowledge, behavioral patterns observed in captive breeding of clouded leopards may have reinforced reproductive isolation [22–24]. Similar geographic isolation likely influenced the evolutionary history of other species in the region. For example, Sumatran (*P. pygmaeus*) and Bornean (*P. abelii*) orangutans, which diverged 1.1–1.7 MYA, are sufficiently differentiated to be considered different species [25–27].

Beyond the clear distinction between *N. n. nebulosa* and *N. n. diardi* individuals in our analyses, there was no indication of further subdivision within these two geographic regions. These findings have important conservation implications because field studies indicate that the Taiwan subspecies (*N. n. brachyurus*) may be extinct [28]. Our analysis of *N. n. brachyurus* was based upon two modern samples of zoo animals of possible

Taiwanese origin and one museum sample. The two zoo samples had a haplotype (NEB-3) found in mainland *N. n. nebulosa* individuals (Figures 1B and 1C; Figures S1 and S2). The short sequence from the *N. n. brachyurus* museum sample was slightly divergent and may represent an historic Taiwanese clouded leopard haplotype. This provides initial support for the introduction of clouded leopards from mainland populations as a management tool for recovery of Taiwan's population.

There are three caveats that temper our conclusions for species-level designation of *N. n. diardi*. First, our sample size for *N. n. diardi* of only three individuals is small, reducing confidence that diversity in Borneo was adequately sampled. Second, clouded leopards may be present on other islands, and these must be sampled to confidently characterize clouded leopard evolutionary history. Third, a morphological assessment of these genetic partitions has not been completed to date. However, it is likely that a 1–1.5 MY separation could have led to morphological differences that would support the molecular genetic findings.

However, the notable distinctions between *N. n. nebulosa* and *N. n. diardi* are based on multiple genetic markers, and if replicated with broader sampling, they would justify the recognition of two distinct clouded leopard species (and the naming of a new felid species). The substantial and cumulative evidence presented here from several distinct molecular markers includes: (1) reciprocal monophyly indicated by mtDNA (771 bp, Figure S3), nuclear DNA (3088 bp; Figure S4), and microsatellite variation (51 loci; Figure 1D and Figure S5–S8) and comparable in magnitude to the species-level divergence among well-accepted *Panthera* species; (2) significant sequence distance between *N. n. diardi* and *N. n. nebulosa* specimens (Table 1; Table S3); (3) fixed nucleotide sequence and haplotype differences between subspecies (Figures S1 and S2); (4) recapitulation of genetic separation by AMOVA and STRUCTURE analyses of microsatellite distinctions; (5) large within-species microsatellite variance (twice that in *Panthera* species, Table 2) with twenty (of 51) displaying nonoverlapping microsatellite allele size ranges between *N. n. nebulosa* and *N. n diardi* (Figure S9); (6) fixed cytogenetic distinctions in chromosome F3 (Figure 2); and (7) a coalescent date of 1.41 MY for divergence between *N. n. nebulosa* and *N. n. diardi*; this number is more than twice the within-species divergence detected between any pair of *Panthera* species [29–34] and is within the same range (1–3 MY) as species-level distinctions across *Panthera* [1]. Taken together, these measures support species-level distinction.

Supplemental Data

Supplemental Data include Experimental Procedures, five figures, and four tables and are available online at: http://www.current-biology.com/cgi/content/full/16/23/2371/DC1/.

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