

Effects of Pleistocene glaciations and rivers on the population structure of Bornean orangutans (*Pongo pygmaeus*)

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Sundaland, a tropical hotspot of biodiversity comprising Borneo and Sumatra among other islands, the Malay Peninsula, and a shallow sea, has been subject to dramatic environmental processes. Thus, it presents an ideal opportunity to investigate the role of environmental mechanisms in shaping species distribution and diversity. We investigated the population structure and underlying mechanisms of an insular endemic, the Bornean orangutan (*Pongo pygmaeus*). Phylogenetic reconstructions based on mtDNA sequences from 211 wild orangutans covering the entire range of the species indicate an unexpectedly recent common ancestor of Bornean orangutans 176 ka (95% highest posterior density, 72–322 ka), pointing to a Pleistocene refugium. High mtDNA differentiation among populations and rare haplotype sharing is consistent with a pattern of strong female philopatry. This is corroborated by isolation by distance tests, which show a significant correlation between mtDNA divergence and distance and a strong effect of rivers as barriers for female movement. Both frequency-based and Bayesian clustering analyses using as many as 25 nuclear microsatellite loci revealed a significant separation among all populations, as well as a small degree of male-mediated gene flow. This study highlights the unique effects of environmental and biological features on the evolutionary history of Bornean orangutans, a highly endangered species particularly vulnerable to future climate and anthropogenic change as an insular endemic.

Asian great ape | genetic structure | radiation | geographical barriers | sociobehavioral barriers

Environmental mechanisms are some of the most important forces affecting the evolutionary history and current distribution of species. Such mechanisms have been invoked to explain genetic structure in many temperate European and North American species but with little focus on hotspots of biodiversity and endemism in the tropics (1), where the forces underlying patterns of genetic diversity and differentiation are especially intriguing.

The tropical Asian hotspot of Sundaland is remarkable in that it has been subject to dramatic geological and environmental changes (2, 3). This now partly submerged continental shelf encompasses the Malaysian peninsula, the islands of Borneo, Sumatra, Java, and possibly Palawan (2). It is a historically dynamic tectonic area that underwent notable landmass configuration changes (3). More recently, it has been severely affected by the Pleistocene climatic oscillations (4) of the Quaternary. Changes in sea levels resulted in the cyclical exposure of the continental shelf and the formation of land bridges between the islands (4, 5), allowing for species interchange with subsequent isolation (6). Moreover, climatic fluctuations were accompanied by vegetation changes (2, 7, 8), with shifts in the range and elevational distribution of rainforests. Thus,

these changes led to habitat expansions or contractions, leading to new openings or barriers to gene flow. The Pleistocene was further punctuated by intense regional climatic and habitat changes through extraordinary volcanic eruptions, especially of Mount Toba (9, 10). Finally, Sundaland contains many interesting topographical features, including rivers, lakes, and mountains (5, 11, 12), that may have acted as barriers to dispersal for a number of species, adding yet another potential allopatric force.

The roles of these environmental forces in driving biotic diversity and endemism remain underexplored, particularly in Borneo, the world's second largest tropical island as well as the easternmost Sunda region abutting the Wallace line (13, 14). Its unusually high species endemism (14–16) suggests a combination of specialized ecological niches, refugia formation, and long periods of isolation.

Among the species endemic to the island are the Bornean orangutans (*Pongo pygmaeus*). This rainforest canopy-bound species with an unusually slow life history is characterized by a rich spectrum of genetic, morphological, and cultural variation (17–19). Fossils indicate a much wider distribution of orangutans during the Pleistocene extending from Southern China and Vietnam to Java (11, 18), but orangutans are currently only found, as distinct species, in Borneo (*P. pygmaeus*) and Sumatra (*Pongo abelii*). The ancestors of orangutans therefore probably migrated from the mainland to Sumatra and from there to Borneo (12), yet it remains unclear when and how these colonization events took place.

It is also unclear how the exceptional environmental features of Sundaland, combined with the characteristic behavioral and ecological traits of orangutans, have shaped their phylogeography. For instance, isolation in refugia or through riverine barriers have been described as important forces underlying the genetic structure of some of the African great apes (20–22), yet the evolutionary history of orangutans remains unresolved. First, the high genetic differentiation between Bornean and Sumatran orangutans (17, 23) is intriguing given recurrent land bridge formation between the islands during the Pleistocene glacial periods (5). Second, within Borneo, arguments for a stable distribution since colonization (24) clash with that of a bottleneck possibly associated

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characteristics, are not reciprocally monophyletic, and should therefore be reconsidered.

The surprisingly recent radiation of a single Bornean lineage calls for a more detailed exploration of Bornean phylogeography. We generated an mtDNA phylogenetic network (Fig. 1C), more appropriate for population level studies than phylogenetic trees as they do not force possible ancestral haplotypes to the tips (32, 33). The network revealed seven main star-like geographical clusters, reflecting considerable structuring within the different subspecies. These seven clusters were further supported by a spatial analysis of molecular variance (SAMOVA), which defines groups of populations that are “geographically homogeneous and maximally differentiated from each other” (34). The analysis indicated that among-group variance asymptotes at 79.27% ($F_{CT} = 0.793$, $P < 0.01$) with seven groups of populations. The grouping corresponds to an almost complete separation of all sampled sites except for: (i) Danum Valley (DV), which clusters with South Kinabatangan (SK), a site in close proximity (approximately 90 km) not separated by geographical barriers (Fig. 1B); and (ii) Gunung Palung (GP), clustering with Sabangau (SA), a site with which it shares its only haplotype. Our results point to strong interpopulation differentiation for mtDNA, as corroborated by the high and significant Φ_{ST} values for all 36 population pairs (Fig. 2B). The exceptions are three lower, albeit still significant, Φ_{ST} values between the sites that share haplotypes. Given the heavy reliance of Φ_{ST} and other classic moment-based estimators on intrapopulation diversity (35), we also computed population average pairwise differences (Table S1). We found generally higher levels of diversity between populations than within, providing additional support for interpopulation differentiation.

Microsatellite Analyses. We also examined differentiation patterns using nuclear loci, which are biparentally inherited and therefore representative of both male and female histories, for the seven sites for which we could generate microsatellite genotypes. Both cluster analyses with Structure and significant pairwise population F_{ST} values indicate strong structuring of these sites (Fig. 2), particularly when separated by rivers (Fig. 1B). The structure runs for all seven sites using 12 microsatellite loci (dataset II, Fig. 2A) yielded the highest probability runs for $K = 7$ [Log likelihood (LnL), $-9,619.88$], partitioning each of the sites as a distinct cluster. Likewise, a more detailed analysis for the five sites for which 25 microsatellite loci were available (dataset I) also led to each one being inferred as a separate cluster (Fig. S1). Generally, high pairwise F_{ST} and level of structuring of populations is congruent with our mtDNA results. However, the cluster analyses using nu-

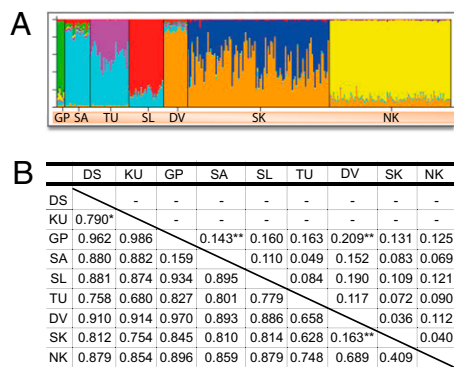


Fig. 2. Population structure based on nuclear microsatellite markers. (A) Structure run for the seven study sites with 12 microsatellite marker data (dataset II) at $K = 7$ (LnL, $-9,576.8$). (B) Interpopulation differentiation with pairwise F_{ST} estimates are above the diagonal and pairwise Φ_{ST} estimates are below the diagonal. All are significant at $P < 0.001$ except when indicated (* $P < 0.05$; ** $P < 0.01$).

clear loci indicate some heterogeneity within populations. As haplotype sharing is rare among populations exchanging migrants, the low levels of gene flow are most likely male-mediated.

We investigated the signature of sex-specific demographic processes more directly by comparing isolation by distance patterns for the nuclear and mtDNA loci. The Mantel test for the relationship between genetic and Euclidean geographical distance yielded a significant and positive correlation for both the nuclear markers and mtDNA (F_{ST} , $r = 0.415$, $P < 0.05$; Φ_{ST} , $r = 0.357$, $P < 0.05$). We also explored the effect of rivers in a partial Mantel test of the association between genetic and cost path distances while controlling for Euclidean distance. Results were significant for the mtDNA ($P < 0.01$; $r = 0.403$) but not the nuclear markers ($P = 0.633$; $r = -0.096$). It is noteworthy, however, that for the mtDNA, only three of the 36 population pairs studied have low Φ_{ST} values (< 0.6). Therefore, most populations are highly differentiated from each other despite the short geographical distances between them.

Discussion

We investigated the evolutionary history of Bornean orangutans using the most comprehensive Bornean sample set compiled to date to our knowledge. Our mtDNA results indicate a surprisingly recent origin for current Bornean populations, and together with the nuclear markers, illustrate that their current distribution has been uniquely shaped by a combination of historical, geographical, and sociobehavioral factors.

Historical Factors: Recent Radiation of Bornean Populations. The recent coalescence of Bornean orangutan haplotypes in the Middle to Late Pleistocene is in striking contrast with that of the other Bornean canopy-bound rainforest species for which data are available, the gibbon *Hylobates muelleri*. This gibbon, distributed throughout the north, east, and west of Kalimantan, has a time to the most recent common ancestor (TMRCA) of 1.78 Ma (95% CI, 1.33–2.25) (36), suggesting that Bornean gibbons have been differentiating within the island for much longer than orangutans. Moreover, Sulawesi macaques (genus *Macaca*), whose ancestors dispersed from Borneo, have a TMRCA with their Bornean sister species of approximately 2 Ma (37). Although the exact timing of their migration is uncertain, the older mtDNA coalescence dates for both Bornean gibbons and Bornean and Sulawesi macaques suggests they have been in Borneo as far back as the Early Pleistocene. Therefore, it is conceivable that orangutans also arrived in Borneo around the same time. Yet, current Bornean orangutan mtDNA haplotypes stem from a very recent common ancestor originating in the Middle to Late Pleistocene.

The relatively short time to the most recent common ancestor of Bornean haplotypes is particularly striking given the deep Bornean–Sumatran orangutan coalescence approximately 3.5 Ma. Such a long differentiation between Bornean and Sumatran haplotypes appears hard to reconcile with the recent episodes of interconnectedness between the islands during the Pleistocene glaciations, most notably during the Last Glacial Maximum approximately 17 ka (2, 5). However, the presence of land bridges does not necessarily imply suitable conditions for migration. A savannah corridor (8) combined with riverine barriers dissecting the exposed land (5, 11) would have presented severe obstacles to migration for orangutans, restricting them to riverine forest galleries along the banks. Coalescence for Bornean and Sumatran haplotypes is expected to vary across species, reflecting differences in dispersal abilities, habitat requirements, or ancestral effective population size, aside from possible discrepancies in dating methods (38). For instance, the south Bornean gibbon *Hylobates albibarbis* and the Sumatran–Malaysian gibbon *Hylobates agilis* have a TMRCA of 1.56 Ma (36), and Bornean and Sumatran pig-tailed macaques have one of 3 to 4 Ma (37). By contrast, the Bornean–Sumatran common ancestor of both the silvered langur

(39) and clouded leopard (40) is much more recent than that of orangutans, gibbons, and pig-tailed macaques, probably because of a higher flexibility in habitat use.

Assuming that orangutans arrived in Borneo around the same time as gibbons and macaques, the recent coalescence of Bornean orangutans could be explained by a bottleneck through a severe rainforest contraction. Such a bottleneck would have had a more dramatic impact on the mtDNA structure of orangutans compared with other species as a result of their low densities and slow life histories (18) as well as habitat requirements. Gibbons were apparently not affected by habitat changes as harshly, perhaps because populations can survive in smaller patches. Our findings are consistent with the survival and expansion of a single lineage from within a refugium in Borneo. Geomorphological and palynological data indicate the presence of dryer, more open vegetation in southern and western Borneo during the last glaciation (2, 41), and by extrapolation also during other glaciations (but c.f. refs. 42, 43). Climate change was especially severe during an extended cold period within the penultimate glaciation between 130 and 190 ka (44, 45), which occurred approximately at the time of mean coalescence of Bornean mtDNA haplotypes. More recently, the last Toba eruption approximately 74 ka resulted in a short, albeit significant, decrease in regional temperatures, ensued by a 1,800-y cold stadial (9, 10). Our data do not provide clear signals to make conclusive statements about potential Toba effects. Nonetheless, the coldest period of the penultimate glaciation (44, 45) was more prolonged than the cold period following the last Toba eruption, suggesting more severe effects of the former on the extent of rainforest across Sundaland. In any event, suitable rainforest habitat for orangutans should have existed in certain regions in Borneo where a refugium population survived the dry glacial conditions. Possible Pleistocene refugia in Borneo have also been described for numerous other rainforest species such as termites, ants, orchids, oaks, and large-bodied mammals (37, 46–51), and together with the isolation of the island, could act as a mechanism of evolutionary diversification driving high Bornean species endemism. Following the expansion of orangutans throughout the island, the Pleistocene climatic oscillations should have led to recurrent population expansions and contractions.

Geographical and Sociobehavioral Barriers. Despite the recent common ancestry of Bornean populations, our analyses revealed high and significant mitochondrial differentiation, with populations within currently recognized subspecies generally displaying as much differentiation as those between subspecies. Of notable interest is the great extent of subdivision and lack of reciprocal monophyly for the morphologically recognized subspecies *P. p. morio* and *P. p. wurbii*. MtDNA haplotype sharing is uncommon and for populations separated by rivers occurs only in two instances: (i) for SA and GP and (ii) for the northern and southern populations across the Kinabatangan river. In both cases, very recent common ancestry could explain the incomplete mtDNA lineage sorting. For North Kinabatangan (NK) and SK, Jalil et al. (27) proposed an expansion from a recent common refugium further west in Mount Kinabalu, as posited for other Bornean species (46, 47, 49). DV, with its low haplotype diversity, might also be the result of a recent range expansion. GP is located proximally to the Bangka–Belitung–Karimata–Schwaner divide, from where orangutans are presumed to have dispersed to the rest of Borneo (12) and where we might expect a rich haplotype diversity. However, the presence of only one mtDNA haplotype shared with populations further east suggests that the current population in GP is recent and/or underwent a severe recent bottleneck. This and other local bottlenecks make it impossible to reconstruct a colonization of Borneo through the southwestern “choke point” (52).

The rarity of mtDNA haplotype sharing among Bornean populations contrasts with patterns in the patrilocal chimpanzees

and bonobos (53, 54), where mtDNA sharing is extensive. Interestingly, two orangutan haplotypes from one site (Tuanan, TU) that were more closely related to those of another site (Sungai Lading, SL) pertain only to males. Although nuclear differentiation among the orangutan populations is significant, we find evidence for a small degree of nuclear gene flow, suggesting that it is male-mediated. Furthermore, the effect of rivers on the isolation by distance patterns for the mtDNA indicate that these are important barriers to female movement, probably as a result of smaller dispersal distances of females (18). An association between mtDNA genetic distance and distances around rivers has also been found in gorillas (20), and a role for differential dispersal distances between the sexes has been posited for western lowland gorillas (55). Our results are consistent with the pattern of female philopatry and male-biased dispersal proposed by Delgado and van Schaik (18) and indicate that the orangutan sexes are subject to very different constraints on mobility. Although female philopatric behavior may be responsible for the strong effect of geographical barriers on mtDNA structure, we cannot make any conclusive statements on the effects of rivers on males. More continuous sampling, especially along rivers and examination of Y-chromosomal markers, representative of male histories, will prove useful in determining how geographical barriers differentially affect the sexes. In addition, further geomorphological data on river course and width changes through time would contribute to the understanding of their vicariant action.

Bornean orangutan distribution and population structure have been uniquely shaped by the Pleistocene fluctuations and by sociobehavioral and geographical barriers to movement. Our findings support a recent radiation of Bornean orangutans in the Middle to Late Pleistocene, resulting in “static” clusters of females strongly separated by geographical barriers and subject to high differentiation, with more mobile males exerting a homogenizing influence on the nuclear gene pool. Further sampling will help establish whether there is a marker specific pattern of clusters versus clines resulting from sex-biased dispersal (c.f. ref. 52). In addition, in depth population genetic studies of other endangered and endemic taxa such as the Bornean gibbons and Sumatran orangutans will be of interest in contrasting the differential effects of environmental processes.

Materials and Methods

Samples and Datasets. Our data comprise noninvasively collected fecal and hair samples from a number of long-term study sites: Gunung Palung (GP), Sabangau (SA), Sungai Lading (SL), Tuanan (TU), Danum Valley Conservation Area (DV), and the Lower Kinabatangan Wildlife Sanctuary (Fig. 1B). We partitioned the latter site into South Kinabatangan (SK) and North Kinabatangan (NK), given the significant differentiation between the locales found by Goossens et al. (28). In addition, we incorporated scattered samples from Warren et al. (17) (Table S2), encompassing most of the current distribution of *P. pygmaeus* (Fig. 1B). Depending on sample quality and data availability, we used two different datasets for mtDNA analyses, and two for nuclear microsatellite analyses (Table S3). DNA extraction and quantification procedures are described in *SI Materials and Methods*.

mtDNA Analyses. Based on unique microsatellite genotypes or mtDNA haplotypes (*SI Materials and Methods*), we obtained the following long-term study site sample sizes: SA ($n = 23$), SL ($n = 26$), TU ($n = 30$), and DV ($n = 18$). We also sequenced low DNA quantity samples from GP ($n = 20$), where individual identification was done through long-term observational data. Additionally, haplotypes for individuals from SK ($n = 38$) and NK ($n = 35$) were from Jalil et al. (27) (GenBank accession numbers EU547189–EU547201). Finally, we resequenced 21 extracts from the Bornean samples in Warren et al. (17) to cover the same region of mtDNA (Table S2). We sequenced a 323-bp region of the mtDNA hypervariable region I (HVRI). Details on the primers and PCR conditions and raw data analyses are provided in *SI Materials and Methods*. Summary statistics including haplotype diversity (h_d), nucleotide diversity (π), and average pairwise differences were calculated in DNAsp 5 (56) and Arlequin 3.11 (57). We conducted model selection tests on jModelTest 0.1 (58, 59), using the Akaike information criterion to choose the most suitable model and its parameters.

For the phylogenetic analyses, we incorporated HVRI haplotypes from all long-term study sites as well as Warren resequenced samples (Tables S2 and S3). First, to infer the coalescence date for Bornean mtDNA haplotypes, we used a Bayesian Markov chain Monte Carlo analysis as implemented in BEAST 1.5.4 (60) and produced a phylogenetic tree. We included the collapsed haplotypes from 211 Bornean and six Sumatran orangutans, as well as 19 humans as an outgroup. Based on the Akaike information criterion from jModeltest, we selected the HKY + G model. We used an uncorrelated relaxed log-normal clock (61), specifying a normal distribution with a mean HVRI substitution rate of 0.1643 substitutions per nucleotide per Myr for the mean rate prior. We chose this corrected HVRI estimate (62) because it takes into account the effects of purifying selection on the entire mtDNA molecule as well as saturation factors affecting the molecular rate decay described in numerous studies (38, 63, 64), and is therefore appropriate for population-level analyses (62, 65). The 95% confidence interval for the normal distribution spanned HVRI substitution rates obtained in other studies, from 0.06 to 0.25 substitutions/site/Myr (66). Using the birth-death prior for branching rates, we carried out two runs for 25 million generations with parameter sampling every 1,000 generations. Tracer 1.4.1 (67) was then used to examine whether the 10% burn-in period and effective sample sizes were adequate. Both runs were combined in LogCombiner 1.4.8, and the resulting tree visualized and edited using Figtree 1.2 (68), omitting human haplotypes. Second, to infer the coalescence date for Bornean and Sumatran mtDNA haplotypes, we used the same procedure, but instead of the corrected mutation rate, we chose two fossil based divergence estimates as priors. Fossil calibration points provide estimates of phylogenetic rates suitable for analyses at the inter-specific level (65). The two calibration points were the Ponginae-Homininae divergence at approximately 14 Ma (69, 70) and the *Pan-Homo* divergence older than 6 Ma (71, 72). We specified log-normally distributed priors, appropriate for paleontological data (73). For the Ponginae-Homininae divergence, we used a log-normal mean of 0, log-normal SD of 0.56, and offset of 13 Ma, thereby obtaining a broad distribution with a 95% interval from 13.4 to 20 Ma. This range incorporates the uncertainties associated with the upper bound estimate of a split. For the *Pan-Homo* calibration, we used a log-normal mean of 0, log-normal SD of 0.56, and offset of 5 Ma, spanning a 95% interval from 5.4 to 7.5 Ma. The tree topology remained the same as in the first analysis, so it is not presented. Third, we investigated phylogenetic relationships at the intraspecific level by generating a median-joining network for the Bornean haplotypes using Network 4.0 (74).

For the population structure analyses, we used data from the long-term study sites GP, SA, SL, TU, DV, NK, and SK. In addition, we incorporated Danau Sentarum (DS) and Kutai (KU) sampling sites from Warren et al. (17) for which at least three samples of precise origin are available (cf. ref. 20; Table S2). We calculated pairwise Φ_{ST} values in Arlequin, using the Tamura Nei model (75) and a γ distribution shape parameter of 0.344. We obtained significance levels using 10,000 permutations. To define the most differentiated groups of populations, we also performed a spatial analysis of molecular variance (SAMOVA) with SAMOVA software, version 1.0 (34), using previously published geographical coordinates (17, 76).

Microsatellite Analyses. Microsatellite analyses focused only on samples from long-term study sites GP, SA, SL, TU, DV, SK, and NK. For the low DNA quality and quantity samples from GP, we could obtain genotypes for six individuals. We genotyped samples from all sites except SK and NK using a panel of 25 highly polymorphic nuclear microsatellite markers (28, 77) listed in Table S4, following the protocol given in *SI Materials and Methods*. Additionally, we incorporated previously generated data from NK and SK for 12 microsatellite markers (28), which were part of our panel of 25 markers. We

standardized the data and performed identity analyses as described in *SI Materials and Methods*. After this procedure, we obtained two data sets: (i) dataset I includes 25 markers and 98 individuals from the five study sites GP ($n = 6$), SA ($n = 19$), SL ($n = 26$), TU ($n = 29$), and DV ($n = 18$); and (ii) dataset II includes 12 markers and 295 individuals from seven study sites, including all from dataset I plus NK ($n = 91$) and SK ($n = 106$).

After Bonferroni correction, we found no deviation from Hardy-Weinberg equilibrium, and only four pairs of different loci from two populations showed linkage disequilibrium, which is most likely explained by demographic effects rather than linkage. Also, we found evidence for possible null alleles for one locus in one population. As it was not consistent across populations, we did not exclude this locus from further analyses.

We used Genetix 4.05 (78) to obtain population pairwise F_{ST} values and significance levels. We also performed two separate analyses on Structure 2.3 (79) using the admixture model with correlated allele frequencies, and the Locusprior model, which improves clustering when the signal is weak without spuriously inferring structure if absent (80). We specified a burn-in length of 10^5 followed by 10^6 Markov chain Monte Carlo steps. For each K, we ran the analysis 10 times. In the first analysis, we incorporated the widely distributed seven populations genotyped at 12 microsatellite markers (dataset II). In the second analysis, we further refined our findings focusing on the five populations for which we have genotypes for 25 microsatellite markers (dataset I).

We calculated geographical distance matrices as Euclidean and cost path distances between all study populations. The latter, representing true surface distances circumnavigating riverine barriers, were computed from the Shuttle Radar Topography Mission global Digital Elevation Model, as distributed by ESRI (81). We clipped the Digital Elevation Model to encompass the whole of Borneo and filled sinks to obtain a depressionless elevation model, which was then reprojected into the Universal Transverse Mercator coordinate system with a resolution of 100 m. From this, we constructed a flow accumulation raster and extracted grid cells with values of at least 1,000 to generate a stream order raster following the convention of Strahler (82). We then produced a cost raster by designating areas with flow accumulation values lower than 1,000 and streams of order 1 to 2, a cost of 1, whereas streams of orders 3, 4, and 5 were assigned costs of 3,000, 4,000, and 5,000, respectively. Streams of order 6 to 7 were designated as uncrossable barriers (cf. ref. 20). After masking the resulting cost raster with the Shuttle Radar Topography Mission Water dataset (81), we calculated dyadic cost path distances between the study populations. For all geospatial analyses, we used ArcInfo Spatial Analyst extension for ArcGIS 9.3 (83).

To investigate the association between genetic (pairwise Φ_{ST} for HVRI and F_{ST} for microsatellite markers) and geographical distances (Euclidean and cost path), we performed (partial) Mantel tests in R 2.10.1 (84), using the "ecodist" package (85).

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Supporting Information

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SI Materials and Methods

DNA Extraction and Quantification. We extracted DNA using the QIAamp DNA Stool Mini Kit (Qiagen) following the manufacturer's protocol with one modification: samples were allowed to incubate for a minimum of 30 min before elution. We quantified DNA through real-time quantitative PCR using the protocol from a previous study (1). The real-time PCR assay allows determination of the number of positive PCR replicates per extract necessary to obtain a 99% confidence level that a homozygous genotype is correct (1). For a heterozygous genotype, our criterion was the observation of each of the two alleles at least twice in independent PCRs.

mtDNA Analyses. We sequenced a 323-bp region of the mtDNA HVRI using the primers DLF (5'-CCT GCC CCT GTA GTA CAA ATA AGT A-3') and D5 (2). PCR amplifications were performed in a 20- μ L reaction volume containing 0.25 μ M of each primer, 0.2 mM dNTPs, 1 \times PCR buffer (Qiagen), 2 μ L BSA, 0.5 U HotStarTaq DNA polymerase (Qiagen), and 1 μ L template DNA. PCR conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 45 cycles of 94 °C for 40 s, 52 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 10 min. Reactions were purified with the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's recommendations. Cycle sequencing was performed in a 10 μ L reaction volume containing 1 μ L of purified PCR product, 1 \times sequencing buffer (80 mM Tris, 2 mM MgCl₂, pH 9.0), 0.4 μ M forward primer, and 0.3 μ L BigDye Terminator, version 3.1. The cycle sequencing conditions were initial denaturation at 95 °C for 45 seconds, followed by 30 cycles of 95 °C for 30 s, 52 °C for 20 s, and 60 °C for 2 min. Capillary electrophoresis was carried out using the 3730 DNA analyzer (Applied Biosystems).

All raw data were viewed and edited in Sequencing Analysis 5.2 (Applied Biosystems). The sequences were aligned with ClustalW (3) in Bioedit 7.0.9.0 (4) and collapsed using DAMBE 5.0.7.2 (5). Unique DNA sequences have been submitted to the EMBL database under accession numbers FR717918–FR717940. The resequenced samples from Warren et al. (2) have been updated maintaining former accession numbers.

Microsatellite Analyses. To standardize the data sets from NK and SK, we genotyped at least 10 original extracts from NK and SK for each of the 12 loci. We ran these samples on the same instruments and analyzed them in the same way as for the other study sites. In cases in which NK and SK genotypes were found to differ as a result of bin set discrepancies, we adjusted allele sizes to match our current bin set. We were not able to complete genotypes for all 25 markers as a result of low DNA quantity and quality, probably because of long-term storage degradation.

All samples from all sites were subjected to identity analyses on Cervus 3.0 (6, 7). If identical genotypes were found, only one was included in our data set.

PCR amplifications were performed as multiplex reactions in an 8 μ L volume containing 1 μ L DNA, 4 μ L Multiplex Master Mix (Qiagen), 0.8 μ L primer mix, and 2.2 μ L water. Amplification conditions were: initial denaturation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 90 s, 72 °C for 1 min, and a final extension at 60 °C for 30 min.

We performed capillary electrophoresis on the 3730xl DNA Analyzer (Applied Biosystems). Products were analyzed using GeneMapper version 4.0 (Applied Biosystems).

We used Arlequin 3.11 to calculate deviation from Hardy–Weinberg equilibrium and GenePop 4.0 (8, 9) to assess linkage disequilibrium. We checked for allelic dropout and null alleles using ML-NullFrEq (10).

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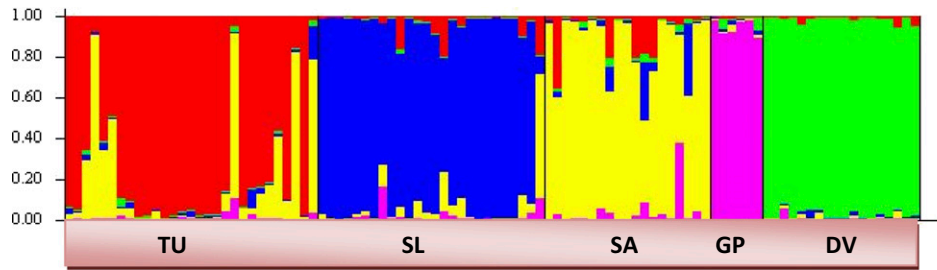


Fig. S1. Structure run for the five study sites with 25 microsatellite marker data (dataset I) at $K = 5$ (LnL, -5,395.4).

Table S1. Population average pairwise differences calculated with Arlequin 3.11

Site	GP	SA	SL	TU	DS	KU	DV	SK	NK
GP	0*	0.86	13.41	12.24	11.81	10.54	8.72	8.95	9.89
SA	0.15	1.42*	14.27	13.23	12.11	11.54	9.64	9.91	10.99
SL	12.63	12.78	1.56*	11.96	12.99	11.82	10.01	10.33	13.35
TU	10.44	10.72	9.38	3.6*	11.28	9.73	6.85	7.61	10.14
DS	13.57	14.57	15.53	14.84	3.51*	10.87	11.7	12.58	15.34
KU	10.02	10.31	10.52	7.42	8.6	1.04*	7.34	8.17	10.72
DV	8.44	8.66	8.96	4.78	9.67	6.55	0.54*	1.71	3.93
SK	7.86	8.11	8.46	4.72	9.73	6.56	0.35	2.18*	3.23
NK	9.07	9.47	11.75	7.52	12.77	9.39	2.84	1.33	1.64*

*Diagonal elements represent average number of intrapopulation pairwise differences. Above this diagonal line are average number of interpopulation pairwise differences, and below are corrected average interpopulation pairwise differences, computed using Tamura-Nei γ distribution with shape parameter 0.344.

Table S2. Samples from the Warren et al. (2) dataset included in analyses after resequencing (unchanged EMBL accession numbers)

Code	Status	Origin/reference	Site assigned
OU TNK41	W	Kutai National Park, EK	KU
OU TNK39	W	Kutai National Park, EK	KU
OU TNK37	W	Kutai National Park, EK	KU
OU TNK36	W	Kutai National Park, EK	KU
OU TP14	R	Tanjung Puting, CK	None
OU TP6	W	Tanjung Puting, CK	None
OU TP24	W	Tanjung Puting, CK	None
OU DSRA	W	Danau Sentarum, NK	DS
OU DSLE1	W	Danau Sentarum, NK	DS
OU DSME1	W	Danau Sentarum, NK	DS
OU DSME2	W	Danau Sentarum, NK	DS
OU SEUA	R	Semongok, Sarawak	None
OU SEBU	R	Semongok, Sarawak	None
OU SEOA	R	Semongok, Sarawak	None
OU SE8	R	Semongok, Sarawak	None
OU KPC	W	Sangatta, EK	None
OU KAI	W	Sangatta, EK	None
OU SB71	W	Sandakan, Sabah	None
OU SB60	W	Kinabatangan, Sabah	None
OU SB57	W	Sukau, Kinabatangan, Sabah	None
OU SB372	R	Sepilok, Sabah	None

Table modified from Warren et al. (2). CK, central Kalimantan; EK, east Kalimantan; NK, north Kalimantan; KU, Kutai; DS, Danau Sentarum; R, rehabilitation center; W, wild.

Table S3. mtDNA and nuclear microsatellite datasets

Marker	Analyses	Samples included
mtDNA	Phylogenetic reconstruction	Resequenced Warren dataset and GP, SA, SL, TU, DV, SK, and NK
mtDNA	Population structure	Resequenced Warren sites KU and DS, and GP, SA, SL, TU, DV, SK, and NK
Nuclear microsatellites	Population structure	GP, SA, SL, TU, and DV (25 microsatellite loci)
Nuclear microsatellites	Population structure	GP, SA, SL, TU, DV, SK, and NK (12 microsatellite loci)

