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Habitat fragmentation and genetic diversity in natural populations of the Bornean elephant: Implications for conservation

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ABSTRACT

The Bornean elephant population in Sabah, with only 2000 individuals, is currently mainly restricted to a limited number of forest reserves. The main threats to the species' survival are population fragmentation and isolation of the existing herds. To support and help monitor future conservation and management measures, we assessed the genetic diversity and population structure of Bornean elephants using mitochondrial DNA, microsatellites and single nucleotide polymorphisms. Our results confirmed a previously reported lack of mitochondrial control region diversity, characterized by a single widespread haplotype. However, we found low but significant degree of genetic differentiation among populations and marked variation in genetic diversity with the other two types of markers among Bornean elephants. Microsatellite data showed that Bornean elephants from the Lower Kinabatangan and North Kinabatangan ranges are differentiated and perhaps isolated from the main elephant populations located in the Central Forest and Tabin Wildlife Reserve. The pairwise F_{ST} values between these sites ranged from 0.08 to 0.14 ($p < 0.001$). Data from these markers also indicate that the Bornean elephant populations from Lower Kinabatangan Wildlife Sanctuary and North Kinabatangan (Deramakot Forest Reserve) possess higher levels of genetic variation compared to the elephant populations from other areas. Our results suggest that (i) Bornean elephants probably derive from a very small female population, (ii) they rarely disperse across current human-dominated landscapes that separate forest fragments, and (iii) forest fragments are predominantly comprised of populations that are already undergoing genetic drift. To maintain the current levels of genetic diversity in fragmented habitats, conservation of the Bornean elephants should aim at securing connectivity between spatially distinct populations.

1. Introduction

The Asian elephant (*Elephas maximus*) once ranged from Mesopotamia in the west across the Indian subcontinent to South-east Asia (including the islands of Sumatra, Java and Borneo) and China and as far north as the Yangtze River (Olivier, 1978). As a consequence of habitat loss and fragmentation, it has been extirpated from approximately 85% of its historical range, and only exists in a number of fragmented and isolated populations in South and South-east Asia (Sukumar, 1989; Fernando and Lande, 2000). As a consequence it is presently classified as 'Endangered' by the IUCN (International Union for Conservation of Nature). Elephants in Borneo are morphologically, and behaviourally distinct from the elephants of mainland Asia (Cranbrook et al., 2008). Also, the genetic distinctiveness of the Bornean elephant from other mainland Asian elephant subspecies makes it one of the highest priority populations for Asian elephant conservation (Fernando et al., 2003). They are

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considered as an evolutionary significant unit, requiring specific conservation measures, however, their recognition as formal subspecies (*Elephas maximus borneensis*) status still awaits more detailed study.

Bornean elephants have a very limited distribution, restricted to approximately 5% of the island, at the extreme northeast, mostly in the Malaysian state of Sabah. They are usually found in family groups (from 5 to 20 individuals) that sometimes merge together to form larger herds of up to 200 individuals (Othman, unpublished data). They are found in five major ranges, with a total of 2000 individuals (95% CI: 1184–3652, Alfred et al., 2010; Elephant Action Plan, Sabah Wildlife Department, 2012–2016, see Fig. 1). The main threat facing the Bornean elephant, as with all mainland Asian elephants, is habitat loss and fragmentation which occurs directly through conversion of existing forests to commercial plantations, such as palm oil, or for permanent human settlement (Elephant Action Plan, Sabah Wildlife Department, 2012–2016). For instance, in the last 50 years, 80% of the Lower Kinabatangan floodplain forest has been converted to agricultural land or used for human settlement (Estes et al., 2012; Abram et al., 2014). The remaining forest in this region is now highly fragmented; yet, it has the highest elephant density of the five ranges with 2.15 individuals per km^2 (Alfred et al., 2010; Estes et al., 2012). With increasing elephant density, the risk for human–elephant conflict and associated human and elephant mortality also rises (Santiapillai and Ramono, 1993; Williams et al., 2001; Alfred et al., 2011). Connectivity between ranges (i.e. between Lower Kinabatangan and North Kinabatangan, Lower Kinabatangan and Tabin Wildlife Reserve, Tabin Wildlife Reserve and Central Sabah) is now lacking, although elephants are increasingly traveling through oil palm plantations (Goossens, unpublished data), which could act as corridors, but also increase human–elephant conflicts.

Habitat fragmentation and loss can also affect the genetic structure of populations both directly and indirectly by restricting gene flow or increasing the levels of genetic drift and inbreeding (e.g. Reed and Frankham, 2003). Changes in genetic diversity associated with habitat fragmentation have been found in Bornean orang-utans that share the same habitat with the elephants (Goossens et al., 2005). Orang-utan populations in the Lower Kinabatangan region have experienced a dramatic demographic decline and are undergoing rapid genetic differentiation induced by genetic drift as a consequence of anthropogenic isolation (Goossens et al., 2006; Jalil et al., 2008; Sharma et al., 2012a). Due to severe habitat loss and range contraction, the Bornean elephant may therefore be at risk of deleterious population effects such as loss of genetic diversity, inbreeding depression and ultimately extinction (e.g. Templeton et al., 1990; Saccheri et al., 1998). However, in long-lived species with overlapping generations, such as elephants, signatures of genetic loss may be masked for decades or even centuries. Retaining of genetic diversity for longer time periods due to the low reproductive rate and long generation time means that deleterious effects of habitat fragmentation will take longer to manifest themselves. So documenting such impacts can only be done after a long time period has elapsed (Armbruster et al., 1999; Ewers and Didham, 2006). In order to make biologically sound conservation plans, an understanding of the current amount of genetic diversity remaining in natural populations of Bornean elephant and its distribution among populations is essential. This is also important for improving and informing their future management.

Previous studies have found low levels of genetic diversity in Borneo elephant. Fernando et al. (2003) compared mitochondrial DNA (mtDNA) and microsatellite diversity for Asian elephant populations

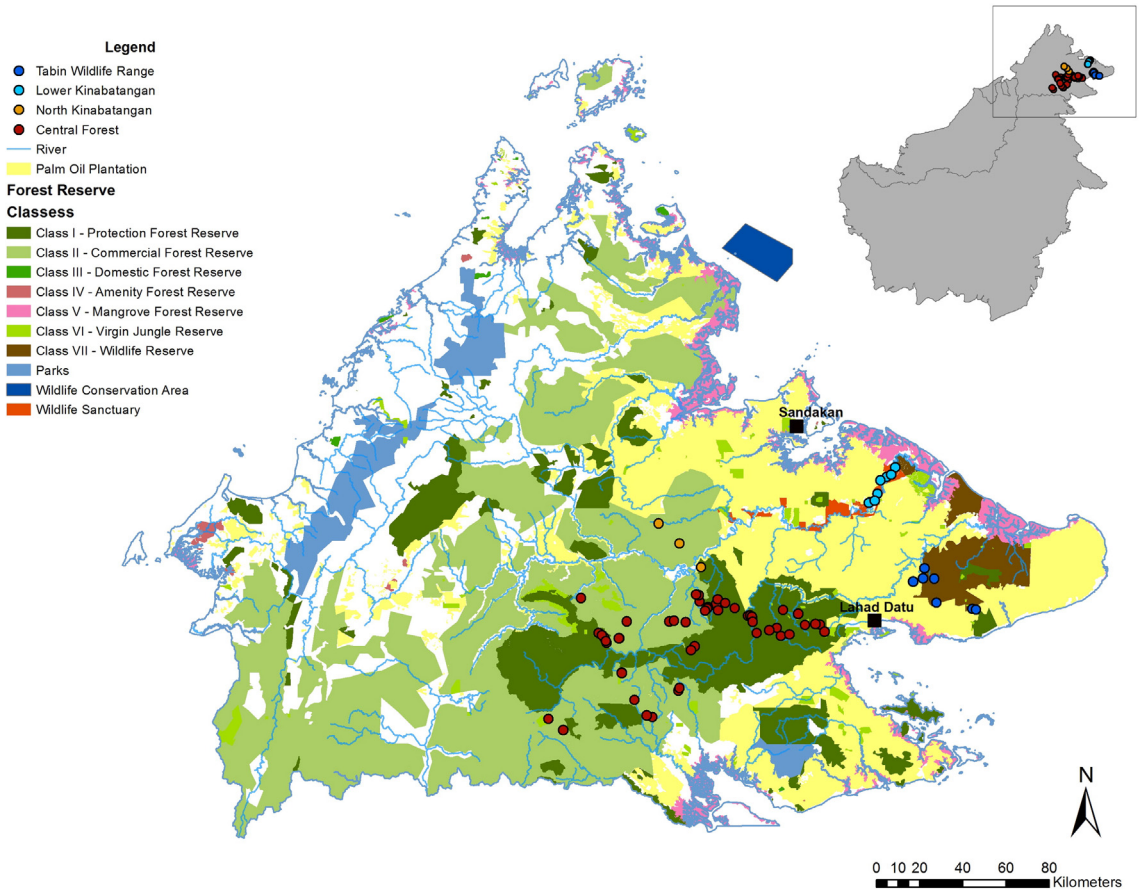

Fig. 1. Map showing locations where Bornean elephant fecal and blood samples were collected. Black squares in the map show the sites where elephant blood samples were obtained (Sandakan and Lahad Datu). The fecal samples were collected from elephants in four main ranges (as described in Material and methods): i) Lower Kinabatangan, ii) North Kinabatangan (Deramakot), iii) Central Forest (Ulu Segama Malua, Kuamut, Gunung Rara, and Kalabakan Forest Reserves, Maliau Basin Conservation Areas), and iv) Tabin Wildlife Reserve.

and found a single mitochondrial haplotype in Bornean elephant samples analyzed from Sabah. They concluded that the Bornean elephant split from other *Elephas* subspecies around 300,000 years ago and was therefore indigenous to Borneo following a Pleistocene colonization. Sharma et al. (2012b) used previously identified set of SNPs and confirmed that Bornean elephants exhibited low genetic diversity. In both studies, however, the sample sizes analyzed were very small, with a total of 20 and seven individuals, respectively.

Therefore, the main aim of the present study was to (i) assess the level of genetic diversity of Bornean elephant and its distribution across the entire range; and (ii) detect possible genetic differentiation between the different local populations in Sabah using a large sample size from across much of the elephant range. It was addressed by using mtDNA and nuclear genetic markers (i.e. microsatellites and SNPs). This will yield insights into the genetic connectivity of remnant populations and indicate which populations are the most genetically isolated and most in need of restoration management (Frankham et al., 2002).

2. Material and methods

2.1. Study sites and sampling

Elephants in Sabah are distributed in five main ranges: i) Lower Kinabatangan, ii) North Kinabatangan (Deramakot, Tangkulap and Segaliud Forest Reserves), iii) Central Forest (Ulu Segama, Malua, Kuamut, Gunung Rara, and Kalabakan Forest Reserves, Danum Valley and Maliau Basin Conservation Areas), iv) Tabin Wildlife Reserve, and v) Ulu Kalumpang (Alfred et al., 2010; Elephant Action Plan, Sabah Wildlife Department, 2012–2016). All ranges (but Ulu Kalumpang, due to its inaccessibility) were covered and systematically searched for elephant feces between October 2005 and November 2007 (Fig. 1). Samples were mainly collected along logging roads where elephants consume grass and travel. The same procedure was conducted for three ranges (Central Sabah, Tabin and North Kinabatangan). For the Lower Kinabatangan population, samples were collected along the main river, in riparian feeding areas where individuals were encountered, allowing collection of fresh samples. Samples were collected during field expeditions of 5–7 days, giving a short time period during which samples were collected from every location. Fresh elephant dungs (less than 1–2 days old) were sampled by collecting approximately 5–10 g of dung from the outermost layer of intact dung boli into a 50 mL Falcon tube filled with 70% ethanol. Dungs of up to 7–10 days old were also sampled in the absence of fresh dung piles. We collected as many samples as we could to ensure that as many different individuals from social groups could be analyzed. The use of microsatellite data would then allow us to discard samples from same individuals. In total, 779 fecal samples were collected across Sabah. GPS coordinates were taken for each sample. Of these 779 samples, 273 were chosen for further analysis. These were fresh feces (between a few hours and two days old), sampled from free-ranging elephants. Out of these 273 elephant feces, 170 were from 14 distinct family groups for which each matriarch was visually identified. The samples used for DNA extraction from each of the forest range were as follows: i) Lower Kinabatangan (LK) ($n = 46$), ii) North Kinabatangan (Deramakot (DER); $n = 33$), iii) multiple sites in the Central Forest (CF) (Ulu Segama-Malua (USM); $n = 78$, Gunung Rara (GR); $n = 32$, Kalabakan (KAL); $n = 46$, Kuamut (KU); $n = 6$, Maliau Basin (MB) Conservation Area; $n = 9$), and iv) Tabin Wildlife Reserve (TWR) ($n = 23$).

Fresh whole blood samples were also collected from 20 Bornean elephants originating from different locations in Sabah between 2009 and 2011. Seven of these samples were collected from elephants in the Lok Kawi Wildlife Park in Sabah and the original source population is known for these samples. Individuals that were sampled in Sandakan and Lahad Datu areas, originated from the main elephant populations in the LK and CF ranges (Fig. 1).

2.2. Molecular analysis

DNA from elephant fecal samples was extracted using the QIAamp® DNA Stool Mini Kit (QIAGEN Ltd., West Sussex, United Kingdom) with modification. Samples were lysed in ASL buffer and an InhibitEX pill was added. The DNA product was dissolved in 200 μL of buffer AE and stored at −20 °C. Two extractions per fecal samples were performed. Genomic DNA was extracted from blood samples using the protocol as described in Sharma et al. (2012b). The extracted DNA from each sample was analyzed using three different markers: a) Microsatellites, b) Single nucleotide polymorphisms, and c) Mitochondrial DNA. The total number of samples analyzed for each marker type is not the same (see Table 1).

2.2.1. Microsatellites

Eighteen dinucleotide microsatellite loci isolated by Kongrit et al. (2007) for the Asian elephant were used to genotype 273 DNA samples and 224 unique elephant individuals were identified. All forward primers were fluorescently labeled and combined in multiplexed polymerase chain reactions (PCR) (see Table S1) and amplified in 10 μL volumes using the Multiplex PCR Kit containing 5 μL of Multiplex Mix, 1 μL of 10X primer mix, 1 μL of Q buffer, 1 μL of water, and 2 μL of DNA template (QIAGEN). Amplifications for each sample were repeated three times as follows: initial denaturation at 95 °C for 10 min; denaturation at 95 °C for 45 s, annealing at 58 °C for 45 s, extension at 72 °C for 1 min and final extension for 10 min. Amplification was completed after 45 cycles and included negative controls to check for contamination. A positive control was used to standardize our allele scoring. PCR products were sent to Macrogen Inc., South Korea for genotyping. If analysis of three positive PCRs per locus was not conclusive, we performed the analysis of four additional positive PCRs, as in Taberlet et al. (1996). For peak identification and fragment sizing, Peak Scanner version 1.0 (www.appliedbiosystems.com/peakscanner) was used.

2.2.2. Single Nucleotide Polymorphisms (SNPs)

SNP genotypes were obtained for 70 unique individuals chosen from all four main ranges that were also included in the set of elephants genotyped for microsatellite markers and from 20 blood samples (Fig. 1, Table 1). SNP genotyping was performed using the MassARRAY platform from Sequenom (San Diego, CA, USA). Five assays targeting 194 SNPs were selected (Gabriel et al., 2009; Sharma et al., 2012b). Each assay allowed us to co-amplify between 28 and 42 SNP loci and is referred to as “plexes” in the following.

Before genotyping the elephant DNA from fecal samples for all plexes, we established the efficiency of SNP genotyping from fecal samples using the Sequenom iPLEX protocol, and optimized the reactions by genotyping a representative sample of 48 individuals for two plexes to evaluate DNA quality in the fecal samples that were collected and extracted in 2005–2007. The quality of the DNA was also screened through the amplification of one microsatellite locus (amplicon size <100 bp), retaining only samples showing positive amplifications. Total DNA in extractions was measured using a Nanodrop ND-1000 spectrophotometer. The final concentration of the diluted DNA was kept at a working concentration of 10× (~200 ng/μl) according to the iPLEX protocol from Sequenom. PCR reactions were conducted using the extracted fecal DNA concentrations at 1, 2, 5, 10, and 20× (at five different concentrations). For each sample, PCRs were performed in duplicate on independent plates to avoid cross-contamination. Quality control criteria for genotyping were adopted using water as negative control and inter-plate duplicates. The MassARRAY Typer 4.0 software was used for data acquisition and analysis. Genotypes were called after cluster analysis using the default setting of Gaussian mixture model. Genotype calls were then reviewed manually to undo any uncertain calls due to clustering artifact. DNA extracted from elephant whole blood was included as positive controls. For every 96-well sample plate, one well was used as a blank control (water) and five wells as

Table 1
Details of elephant samples used for each type of marker.

Major Bornean elephant range	Sampled site	Analyses for each type of marker			
		Mitochondrial DNA (mtDNA)	Microsatellites (Msat)	Single nucleotide polymorphisms (SNPs)	
		Feces	Feces	Blood	Feces
Lower Kinabatanagan	Lower Kinabatanagan (LK)	5	43	10	20
	Sandakan	–	–	6	–
North Kinabatangan	Deramakot (DER)	–	33	–	9
Central Forest (CF)	Ulu Segama Malua (USM)	25	50	–	7
	Gunung Rara (GR)	8	30	–	8
	Malua Basin (MB)	–	7	–	7
	Kuamut (KU)	–	6	–	–
	Kalabakan (KAL)	–	34	–	9
	Lahad Datu	–	–	4	–
Tabin Wildlife Reserve	Tabin Wildlife Reserve (TWR)	5	21	–	10
–	Lok Kawi Wildlife Park (captive elephants)	4	–	–	–
Total number of samples analyzed for each marker type (n)		47	224	20	70

duplicate checks (using DNA from good quality blood samples). Plex with less than 99.5% concordance in duplicate checks was considered failed and was repeated. Plex with more than 25% call rate in the blank control was also considered failed. MassARRAY typer 4.0 uses a three parameter model to calculate significance of each putative genotype. A final genotype is called and assigned as 'conservative', 'moderate', 'aggressive', 'low probability', and 'user call (manual calls)' based on degree of confidence. SNPs were classified as "failed assays" when the majority of genotypes could not be scored due to low probability or when the samples did not cluster well according to genotype. SNPs that were not found to conform to Hardy–Weinberg Equilibrium (HWE) in one or more samples were cross-checked. Each elephant genomic DNA isolated from fecal and blood samples was amplified in 3–5 different replicates (average 4 replicates for each plex), allowing us to quantify genotyping error rates.

2.2.3. Mitochondrial DNA (mtDNA)

The mtDNA of forty-seven unique elephant individuals from a number of different sites were successfully amplified and sequenced using the published primers (Fernando and Lande, 2000; Table 1). These individuals were identified from each site on the basis of unique genotypes across multiple eighteen microsatellite loci. Fernando et al. (2003) analyzed a 630 bp fragment of mtDNA comprising cytochrome b (109 bp), $tRNA_{thr}$, $tRNA_{pro}$ (135 bp), and the hypervariable left domain of non-coding control region (386 bp), and only one haplotype was detected for all 20 Bornean elephant samples. In order to check for the existence of new haplotypes, we amplified and sequenced the same mtDNA fragment. All PCR amplifications followed Fernando et al. (2003) and each amplicon was sequenced in both directions. Sequencing was performed at DNA Sequencing Core at Cardiff University in an ABI3100 automated sequencer. Sequences for each individual were aligned in SEQUENCHER 3.1.2. All contigs were manually inspected and sequences were compared with published Asian elephant sequences from Fernando et al. (2003) (GenBank accession numbers AY245538, AY245802 to AY245827).

2.3. Data analyses

The two nuclear data sets, microsatellite and SNP loci were treated independently for each analysis. Further, the analysis was repeated by combining the microsatellites and SNPs in an effort to increase statistical power (Morin et al., 2009). In this case, a total of 60 nuclear loci (18 microsatellites and 42 SNP loci) were used in the analysis of 70 individual elephants from four major ranges. We used only 42 out of 194 SNP loci in the data analyses because many putative SNPs were found monomorphic (refer Section 3.1 for details).

2.3.1. Genotyping error

The software GIMLET v.1.3.3 (Valière, 2002) was applied to the microsatellite and SNP data to estimate error rates in individual genotyping: ADO (allelic drop out), and successful PCRs. GIMLET also allows to construct a consensus multilocus genotype (the most likely genotype based on all amplifications of a sample) from a set of PCRs and to calculate genotyping error rates comparing the repeated genotypes and their consensus. Genotypes were validated independently by B.G. and N.O. for microsatellite data and by R.S. and C.K.R for SNP data.

Because we found homozygosity excess in our microsatellite data, we searched for evidence of null alleles using the program MICROCHECKER (Van Oosterhout et al., 2004) and to infer the most probable technical cause of HWE departures. Deviations from HWE due to inbreeding or population substructure should result in heterozygote deficits across most or all loci, whereas technical causes such as null alleles should result in heterozygote deficits that are variable across loci and populations. The frequency of null alleles was also calculated for each locus using FreeNA (Chapuis and Estoup, 2007), with the maximum-likelihood estimation from Dempster et al. (1977). Due to a significant proportion of null alleles found (>10% at any locus) in FreeNA, false homozygote frequencies were used to adjust the number of null alleles by re-naming potential nulls as 999 (Chapuis and Estoup, 2007; Chapuis et al., 2008; Oddou-Muratorio et al., 2009). Further analysis of data used both the adjusted allele frequency data and raw data to assess the effect of null alleles on our results.

2.3.2. Relatedness analyses

We used the information derived from 18 microsatellite loci in 170 individuals (sampled within the family units in each of the site) to estimate pairwise relatedness (r) values using the maximum likelihood method in ML-Relate (Kalinowski et al., 2006). Relatedness estimates were adjusted for the presence of null alleles.

2.3.3. Genetic diversity and population structure

Allele frequencies, mean number of alleles across loci (MNA), observed heterozygosities (H_O), and gene diversity (He) (Nei, 1978) were obtained for microsatellite and SNP data using the GENETIX 4.01 (Belkhir et al., 2000) and GenAlEx (Peakall and Smouse, 2006) softwares. Allelic richness (MNA) was adjusted for discrepancies in sample size by incorporating a rarefaction method, and was estimated for each forest range using Fstat 2.9.3.2 version (Goudet, 1995).

In order to compare parameters inferred from SNPs and microsatellites, we evaluated microsatellite diversity for the same individuals for which we also had SNP genotypes ($n = 70$). These

individuals represented elephant populations from all major forest ranges, i.e. LK, DER, CF, and TWR.

Population structure across the Bornean elephant distribution range was investigated using Bayesian clustering as implemented in STRUCTURE v. 2.3.3 (without spatial information) (Pritchard et al., 2000; Falush et al., 2003), and TESS 2.3.1 (incorporating spatial information) for both SNP and microsatellite datasets (François et al., 2006; Chen et al., 2007). STRUCTURE was used under a model assuming admixture, ignoring population affiliation and allowing for correlation of allele frequencies between clusters. We conducted ten runs for each value of $K = 1$–7 and each run consisted of a 50,000 burn-in followed by 250,000 iterations. The most likely value of K was assessed by comparing the likelihood of the data for different values of K and by the rate of change in the log probability of the data between successive K values (Delta K; Evanno et al., 2005). Structure analyses were repeated after removing all but a few of the individuals that were sampled within the family units. After removing individuals that were related, the final data consisted of 58 individuals from different sites within each of the forest range (LK, $n = 10$; DER, $n = 9$; CF (USM, $n = 9$; GR, $n = 6$; KAL, $n = 8$; KU, $n = 5$; MB, $n = 5$); and TWR, $n = 6$). Structure Harvester was used to calculate and plot Delta K (Earl and vonHoldt, 2011). Assignment of individuals to the inferred clusters was estimated according to the highest q-values (probability of membership). STRUCTURE results were visualized with the program DISTRUCT (Rosenberg, 2004).

TESS was run using the conditional autoregressive (CAR) admixture model with spatial interaction parameter set at 0.6, as recommended by Chen et al. (2007). In the analysis, we also considered other values of the spatial dependence parameter (Ψ) 0.0 and 1.0. This parameter weights the relative importance given to the spatial connectivities ($\Psi = 0$ recovers the model underlying STRUCTURE, while $\Psi = 0.6$ and 1.0 indicate moderate and strong values, respectively). One hundred replicate runs of 100,000 sweeps (disregarding the first 30,000) were performed for K values 2 to 7. The preferred K was selected by comparing the individual assignment results and the deviance information criterion (DIC) for each K (Durand et al., 2009). DIC values averaged over 100 independent iterations were plotted against K, and the most likely value of K was selected by visually assessing the point at which DIC first reached a plateau and the number of clusters to which individuals were proportionally assigned. The 10 runs with the lowest DIC values for the selected K-value were retained and their admixture estimates were averaged using CLUMPP version 1.1.2 (Jakobsson and Rosenberg, 2007), applying the greedy algorithm with random input order and 1000 permutations to align the runs and calculate G′ statistics.

We also applied principal component analysis (PCA) to the microsatellite and SNP genotypes since PCA is not dependent on any model assumptions and can thus provide a useful validation of Bayesian clustering output (Patterson et al., 2006; François and Duran, 2010). We used the R package Adegenet v1.3.4 (Jombart et al., 2008) to carry out standard PCA analysis.

We calculated Wright's F-statistics for both nuclear datasets (SNP and microsatellites) according to the method of Weir and Cockerham (1984) and their significance was tested with 10,000 permutations using the GENETIX 4.01 (Belkhir et al., 2000). We also used Arlequin 3.1 (Excoffier et al., 2005) to perform a hierarchical analysis of molecular variance (AMOVA, Excoffier et al., 1992) to determine significance of genetic variation between the forest ranges and when grouped by geographic location.

In order to test for the impact of null alleles in our microsatellite data set, we also calculated global F_{ST} values with FreeNA software. These values were computed, as described in Chapuis and Estoup (2007), with 10,000 bootstrap iterations, alternatively using and not using the excluding null alleles (ENA) method.

We investigated fine-scale spatial genetic structure by analyzing isolation-by-distance (IBD) using the microsatellite genotype dataset. We performed a Mantel test to investigate the correlation between the Queller and Goodnight relatedness estimator (1989) and geographical distance. The significance of results was assessed by 9999 permutations. This was done using the software GenAlEx (Peakall and Smouse, 2006).

2.3.4. Departures from mutation drift equilibrium

We tested for a departure from mutation drift equilibrium in the microsatellite data using BOTTLENECK version 1.2.0.2 (Cornuet and Luikart, 1996; Piry et al., 1999). Significant departures can be due to changes in population sizes such as expansions and bottleneck under the assumption that samples are obtained from a random mating and isolated population. Under that model bottlenecked populations will show an excess of heterozygotes relative to that expected at equilibrium from observed allelic diversity. BOTTLENECK was run under three mutation models: the infinite alleles (IAM), two-phased (TPM), and stepwise mutation (SMM). The TPM was set at 95% stepwise mutation model and 5% multi-step mutations, as recommended by Piry et al. (1999). Wilcoxon signed-rank tests were used to identify heterozygosity excess (Piry et al., 1999).

3. Results

3.1. Genotyping error

In the case of microsatellite markers, out of the 273 samples genotyped, two samples did not yield reliable results and 224 unique genotypes were identified (representing approximately 11% of the estimated elephant population of Sabah). Comparison of the observed genotypes with the distribution of randomized genotypes generated with the program MICROCHECKER (van Oosterhout et al., 2004) suggested that heterozygote deficiency is at least partly due to the presence of null alleles. However, there was no evidence for scoring errors due to stuttering or large allele dropout, as confirmed by a GIMLET analysis. Sixteen loci in CF, while four loci each in LK, TWR and nine loci in DER were not in HWE. Altogether null alleles were present at intermediate frequency for 13 out of 18 microsatellite loci and their frequency estimates ranged from 3% and 20%. The loci that displayed deviations from HWE are listed in Table S2 with, F_{IS} values being significantly different from zero and positive in all four ranges varying from 0.14 in LK to 0.38 in DER (Table 2).

SNPs were successfully genotyped for all blood samples ($n = 20$). We generated 10,397 (97%) genotype calls (obtained out of 10,716 expected calls) from all the blood samples across all the plexes and replicates. We observed 54, 17, 8, and 6% Sequenom calls that were at “conservative”, “moderate”, “aggressive”, and “low probability” levels of confidence (listed in Table S3), respectively. Of the 194 SNP loci, corresponding to 10,716 genotypes, 319 (3%) did not successfully call any bases within our confidence limits and hence were assigned as “no alleles”. In addition, 12% of the genotype calls were noted as “user calls” because these calls were assigned manually by us. Within the SNP data set consisting of 194 SNPs and 20 blood samples, amplification success rate ranged between 93 and 100% per locus and 156 (80%) of the 194 SNPs were found monomorphic and were excluded from all analyses. Three of the remaining 38 loci showed significant heterozygotes excess, thus 35 loci were used in the data analyses. Individual multilocus genotypes in this SNP data set were on average 97% complete.

For the fecal samples ($n = 70$), the tests performed by using different concentrations of extracted DNA showed that the 10 and 20× concentrations could be scored unambiguously without any quality difference in genotype calls. We therefore performed further genotyping of all fecal-extracted DNA at 10× concentration. We obtained 43,354 (71%) genotype calls out of 60,284 expected calls. In total across all plexes, we observed 33, 11, 6, and 13% Sequenom calls were at “conservative”, “moderate”, “aggressive”, and “low probability” levels of confidence, respectively. A high number of

Table 2
Genetic diversity measures in Bornean elephant using: 18 microsatellite loci (a), and combined SNP and microsatellite loci (b). Mean number of alleles (MNA), Allelic richness (AR), and departures from Hardy–Weinberg proportions (FIS).

a. Population	Sample size (n)	Expected heterozygosity	Observed heterozygosity	MNA	AR	*FIS*
Lower Kinabatanagan (LK)	43	0.47	0.41	3.3	3.21	0.14*
Deramakot (DER)	33	0.42	0.26	2.5	2.50	0.38*
Central Forest (CF)	127	0.25	0.18	3.6	2.80	0.27*
Tabin Wildlife Reserve (TWR)	21	0.21	0.14	1.7	1.83	0.36*
All	224	0.34	0.25	2.8	–	0.30*

b. Population	Sample size (n)	Expected heterozygosity	Observed heterozygosity	*FIS*
Lower Kinabatanagan (LK)	20	0.33	0.29	0.12
Deramakot (DER)	9	0.30	0.27	0.11*
Central Forest (CF)	31	0.23	0.21	0.08*
Tabin Wildlife Reserve (TWR)	10	0.22	0.21	0.03
All	70	0.27	0.24	0.09

* Significant.

genotypes (29%) were assigned as "no alleles" due to bad spectrum (Fig. S1 and Table S3). We only made "user calls" in 9% of the cases. SNP genotyping showed a low percentage of positive PCRs, and ranged from 20 to 92% (average across each sample) and 41–78% (average across each plex). Allelic dropout (ADO) ranged between 0 and 37% in 25 samples with highest ADO rate observed in 6 fecal samples (>20%). We also compared genotype error rate of SNPs across different multiplexing levels (28-plex to 42-plex) and found a significant correlation between genotype error rate of SNPs and multiplexing levels in the fecal samples. The genotyping error rate of SNPs across 28-plex and 42-plex observed was 9 and 25%, respectively.

Our results showed that five of the 194 SNP loci generated no nucleotide signal, 130 yielded monomorphic profiles and of the remaining 59 polymorphic loci, 31 were identified as polymorphic in both fecal and blood samples. We therefore found that 7 and 28 loci were only polymorphic in the blood and fecal samples, respectively. Sixteen of the 59 loci showed a significant departure from HWE ($p < 0.001$) with five loci having positive (heterozygote deficit) and the remainder having negative F_{IS} values (heterozygote excess) and one additional locus had many genotypes (>95%) missing. Hence, these 17 loci were excluded from further analyses, leaving a complete SNP data set of 42 reliable polymorphic loci. Individual multilocus genotypes were on average 61% complete in this data set.

3.2. Relatedness analyses

Analysis of pairwise relatedness (r) of all individual genotypes derived from 18 microsatellite loci revealed an average relatedness of -0.005 ± 0.476. However, average pairwise relatedness values between individuals examined within each site were significantly positive in four (USM, GR, KAL, TWR) out of seven populations and r ranged from 0.264 (KAL) to 0.413 (USM). Furthermore, relatedness values between individuals that were sampled within family units from USM, GR, KAL, and TWR also showed very high values ($r = 0.065$ to 0.783). These results are given in Table S4.

3.3. Genetic diversity

3.3.1. Mitochondrial DNA

All 47 fecal samples were successfully amplified and sequenced, and only one haplotype was detected. This haplotype corresponds to the unique β-haplotype BD, as previously reported by Fernando et al. (2003) in the elephant samples collected from three different locations. Thus sequencing additional individuals did not allow us to identify new haplotypes beyond this.

3.3.2. Microsatellites and SNPs

All 18 microsatellites were polymorphic with between two and eight alleles per locus. The mean number of alleles (MNA) per site ranged from 1.7 to 3.6 and a positive correlation between this parameter and sample size ($r = 0.80$) was observed. Observed heterozygosity (average *Ho* across loci) was lower than expected for all sites and ranged from 0.14 in TWR to 0.41 in LK, whereas expected heterozygosity (average *He* across loci) ranged from 0.21 in TWR to 0.47 in LK (Table 2). After correcting the microsatellite data set for null alleles using EM algorithm (as implemented in FreeNA), both the average observed and expected heterozygosity values were higher than the raw data ranging from 0.23 in CF to 0.46 in LK and 0.26 in CF to 0.49 in LK, respectively. Despite this correction, F_{IS} values were still positive and significant in two sites (DER and CF) (Table S5).

Within the SNP data set the percentage of polymorphic loci ranged from 52 to 90%. It was highest in the samples from LK in agreement with the results obtained using microsatellites, where all loci were polymorphic. Most loci were in Hardy–Weinberg equilibrium and while F_{IS} values differed markedly between datasets, they were generally lower (and non-significant) for SNPs than for microsatellites. The average *Ho* and *He* for the SNP data ranged from 0.22 in TWR to 0.31 in DER, and from 0.23 in TWR and CF to 0.32 in DER, respectively (Table 3). While microsatellites provided higher estimates of *Ho* and *He* for elephants in the LK region, average *Ho* and *He* for SNPs were consistently higher in the elephants from DER than those in the LK (Table 3). Interestingly, we found 94% of SNP loci to be polymorphic in the blood samples from LK and only 65% from CF range. However, the two types of marker do not appear to exhibit very different patterns, as Table 2 suggests for the 70 individuals for which we had genotypes derived from both SNPs and microsatellites. This supports the main result of higher genetic diversity in the elephants from the LK region. This again is confirmed by the combined nuclear data set (microsatellite and SNP), which produced similar levels and patterns of genetic diversity in all populations (Table 2). Hence, taken together, our results agreed in suggesting that elephants in the Kinabatangan region (including LK and DER populations) are genetically more diverse than the others.

3.4. Assessment of population structure

Bayesian cluster analysis of microsatellite genotypes in STRUCTURE supported the existence of two genetic clusters. Examination of Ln P (X|*K*) and Delta *K* values also suggested a level of subdivision at $K = 2$. Cluster 1 consisted mostly of individuals from the geographically isolated LK and DER populations, with a few individuals sampled elsewhere also being assigned to this cluster (Fig. 2a). Cluster 2 included individuals from all forest sites located in CF

Table 3
Comparison of genetic diversity indices across SNP and microsatellite data for 70 individuals. Mean number of alleles (MNA) and departures from Hardy–Weinberg proportions (FIS).

Elephant population	*n*	Expected heterozygosity		Observed heterozygosity		MNA		*FIS*	
		Microsatellites	SNPs	Microsatellites	SNPs	Microsatellites	SNPs	Microsatellites	SNPs
Lower Kinabatanagan (LK)	20	0.45	0.29	0.39	0.26	3.2	1.9	0.13	0.11NS
Deramakot (DER)	9	0.24	0.32	0.17	0.31	2.0	1.7	0.34*	0.03NS
Central Forest (CF)	31	0.23	0.23	0.16	0.23	2.5	1.8	0.29*	0.01NS
Tabin Wildlife Reserve (TWR)	10	0.21	0.23	0.15	0.22	1.7	1.7	0.26	−0.05
All	70	0.28	0.27	0.22	0.25	2.36	1.8	0.26	0.004NS

NS: not significant.
* Significant.

(i.e., USM, GR, KU, MB, KAL), and TWR. Across all individuals, 35% were assigned to cluster 1 with a membership proportion of $q > 0.70$. Individuals within CF and TWR (representing the remaining 65% individuals) were more strongly assigned to the alternative cluster with q varying from 0.75 to 0.95. No further sub-structuring was found within the CF. STRUCTURE analyses performed on the microsatellite data representing only unrelated individuals also produced results similar to $K = 2$ (one cluster comprising LK and DER and the other cluster composed of the other sites).

TESS gave results similar to STRUCTURE, grouping the majority of the individuals from LK and DER together (Figs. 2a and 3), whereas all individuals within the CF and TWR were assigned to another cluster (cluster 1 in Fig. 3). The DIC plot of the TESS runs did not show a well-defined plateau as the DIC values continuously decreased at higher *K*max values (Fig. S2). Across the 10 TESS runs, $K = 2$ showed the most consistent groupings of elephant populations. Higher values displayed less stable clustering and did not recognize additional distinct population clusters aside from the groups inferred at $K = 2$. Also, analyses performed on the data eliminating the related individuals produced results similar to $K = 2$.

The results from the principal component analysis (PCA) of the microsatellite data corroborated the aforementioned analyses. Individual genotypes from the two clusters identified by STRUCTURE and TESS were separated along the first and second components of the PCA, which may provide a slightly better discrimination than the other two analyses. As shown in Fig. 4, the first component which accounted for 16.43% of variation resulted in the separation of LK and DER from the other populations. Simultaneously, the sites located in CF were clustered together with TWR. Furthermore, the second component (accounting for 8.48%) could be interpreted as separating

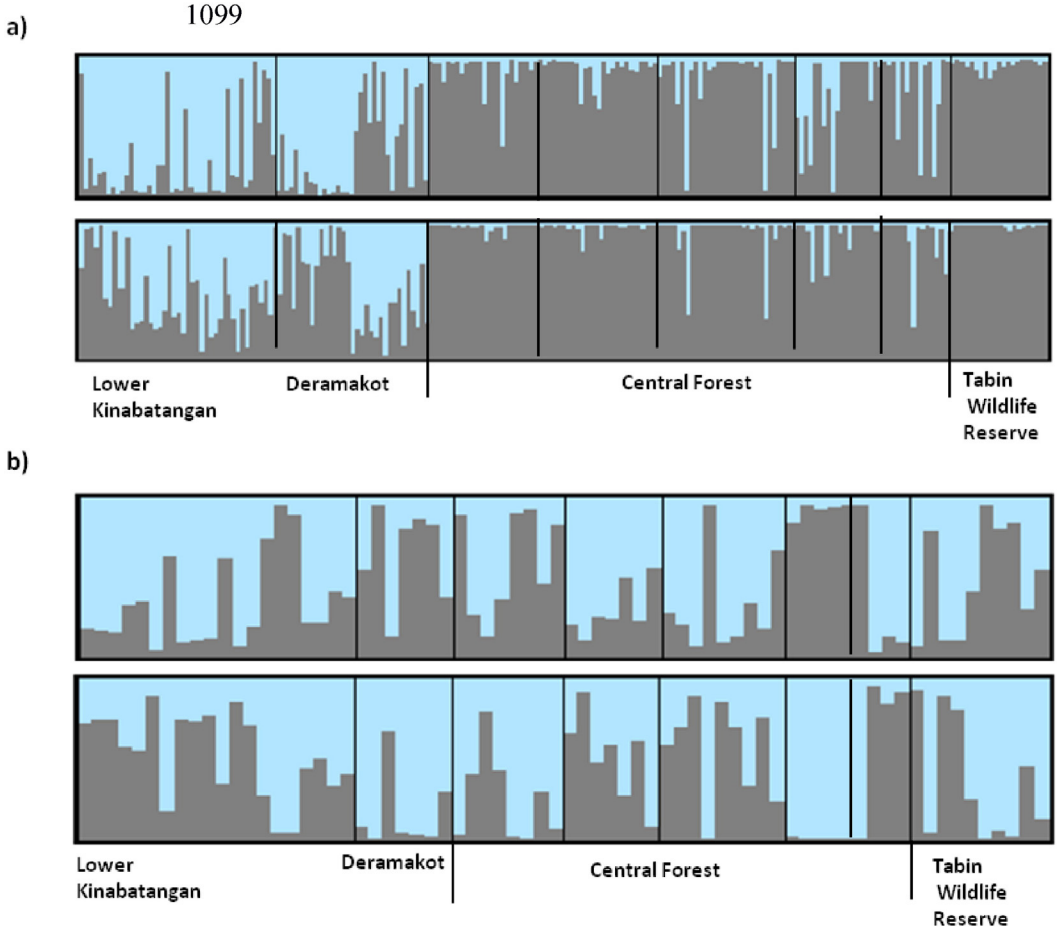

Fig. 2. Individual assignment probabilities of Bornean elephants to genetic clusters using the model-based programs STRUCTURE (above) and TESS (below) run of $K = 2$. Results from a) Microsatellites (for 224 individuals), b) SNPs (for 70 individuals). Each column represents *q* values, the proportions in which a given genotype belongs to a cluster of the given color. Vertical black bars separate sampling sites. Geographic sampling locations are indicated below the figure. Multiple sites were sampled in the Central Forest (Ulu Segama Malua, Kuamut, Gunung Rara, Kalabakan Forest Reserves, and Maliau Basin Conservation Area).

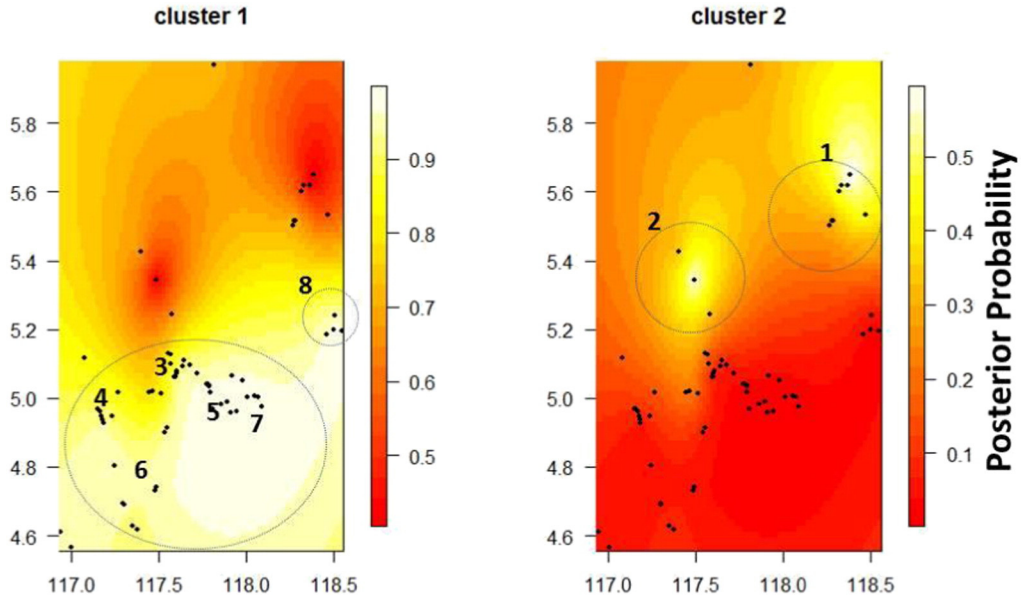

Fig. 3. Spatially explicit predictive map of admixture coefficients as determined by TESS for 2 clusters, $K_{max} = 2$ for microsatellite data. The color scale represents the posterior probability of individuals having membership to a single genetic cluster. Numbered ellipses indicate the populations; 1 = Lower Kinabatangan, 2 = Deramakot (North Kinabatangan), multiple sites in Central Forest: (3 = Ulu Segama Malua (USM), 4 = Gunung Rara (GR), 5 = Malua Basin (MB), 6 = Kuamut (KU), and 7 = Kalabakan (KAL)), 8 = Tabin Wildlife Reserve (TWR). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

LK and DER sites. The overlap between all these clusters further illustrates their weak genetic differentiation. The following principal coordinate axes displayed uninformative clustering patterns.

We also tested the efficiency of our methods for detecting population structure using SNP data. Contrary to the microsatellite results, STRUCTURE detected only a single genetic cluster (i.e., $K = 1$, Fig. 2b). All assignment percentages for the SNP dataset were lower than for the microsatellite dataset. TESS detected the presence of two very weak spatial genetic clusters (Fig. 2b). The PCA approach based on allele frequencies also showed results similar to STRUCTURE and failed to reveal the presence of the two clusters (Fig. 4).

Combining SNPs and microsatellites for 70 individual samples, which would be expected to increase statistical power over either single data set, did not result in the detection of a clear population structure (Liu et al., 2005).

The consistency of the clusters identified through the Bayesian clustering approach for both microsatellite and SNP data was tested by pairwise F_{ST} analysis. Using microsatellites, we found a considerable level of genetic differentiation between all demographically isolated populations in different ranges (average $F_{ST} = 0.10$, $p < 0.001$) suggesting limited gene flow between adjacent populations of elephants. The pairwise F_{ST} values between the sampling sites ranged from 0.03 to 0.14 ($p < 0.001$) (Table 4). We observed that LK, DER and TWR were the most differentiated (F_{ST} values between 0.08–0.14) forest ranges. Furthermore, in a separate analysis, the sites in the CF range, such as GR, KAL, USM appeared to have low (but significant) genetic differentiation (F_{ST} 0.04–0.07). Global F_{ST} and the F_{ST} values for each locus were similar when calculated with (ENA) and without estimating a null allele correction ($F_{ST} = 0.10$, F_{ST}ENA = 0.11). This suggests that the presence of null allele has limited effect, if any, on our calculations of genetic differentiation. AMOVA analyses revealed that variation within populations, between populations, and among regions accounted for 88%, 7%, and 5% of the total variation, respectively ($p < 0.001$).

SNP data produced low pairwise F_{ST} estimates indicative of little to moderate differentiation (pairwise F_{ST} values between 0.006 and 0.136, $p < 0.05$) but were not significant except in the comparisons involving DER and TWR for which F_{ST} values were above 0.10 and 0.13, respectively. Combined data from both SNP and microsatellite marker types produced low F_{ST} estimates indicative of limited to medium differentiation between subpopulations (F_{ST} values between 0.02 and 0.11).

Results from Mantel test on microsatellite data suggest a significant negative correlation between pairwise relatedness and geographic distances ($r = -0.142$, $p = 0.000$) among elephant populations, implying that relatedness decreased with geographic distance.

3.5. Departures from mutation drift equilibrium

For microsatellites, the results of the BOTTLENECK analysis showed that there was no consistent or strong signal for a departure from mutation drift equilibrium. Only for the DER sample we found a significant departure under three mutational models (Wilcoxon test; TPM: P = 0.00008; IAM: P = 0.00002, SMM: P = 0.00134). The LK population showed significant excess of heterozygotes only under the IAM and TPM (IAM: P = 0.00032; TPM: P = 0.01184). None of the other population showed evidence of heterozygosity excess.

4. Discussion

4.1. Genetic diversity and assessment of population structure

Our study revealed relatively low levels of genetic variation in Bornean elephants, using both mitochondrial sequence and nuclear genetic markers. The lack of mtDNA variability in Bornean elephant was not surprising as the only previous study by Fernando et al. (2003) found no variation in this region of mtDNA across 20 individuals. MtDNA studies in other Asian elephants have shown that adult females of a family or a social group share the same haplotype and are closely related to one another due to the matriarchal social system of elephants (Fernando and Lande, 2000; Vidya et al., 2005a; de Silva et al., 2011). For instance, study by Vidya et al. (2005a) found only one haplotype in the world's largest Asian elephant population (of over 9,000 elephants) in the Nilgiris, southern India. Therefore, the fixation of a single mtDNA haplotype among Bornean elephants is probably more a consequence of a recent or an ancient population bottleneck (Fernando et al., 2003). The current study, however, significantly expands the geographic and demographic importance of these findings, suggesting that an

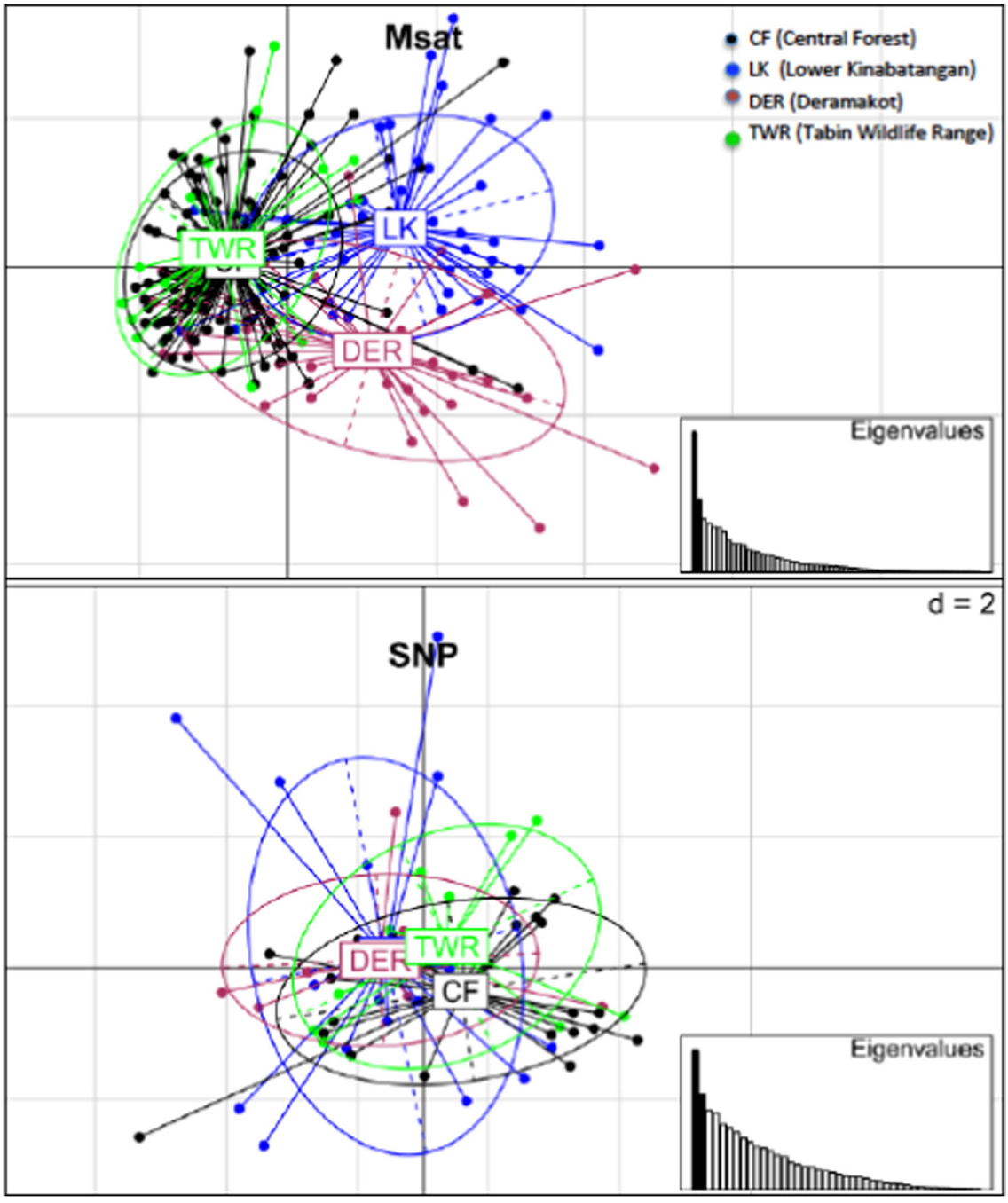

Fig. 4. Principal component analysis (PCA) of the Bornean elephant fecal samples based, a) microsatellite (Msat), b) SNP. Dots represent different individuals; bottom right inset shows eigenvalues of principle components in relative magnitude.

exceptionally high proportion of Bornean elephants in Sabah have a single mtDNA lineage regardless of their present geographic location.

Our microsatellite and SNP data based on larger geographic coverage across Sabah and increased sample sizes confirm earlier findings of low levels of genetic variation in Bornean elephant. Fernando et al. (2003) compared all Asian elephant populations (e.g. Borneo, Cambodia, Bhutan, Bangladesh, Thailand, Sumatra, Sri Lanka, and South India; *He* = range 0.014–0.63 for five microsatellite loci) and showed that

Table 4
Pairwise FST values between the sampling sites for microsatellite data. The pairwise FST value between the two major genetic clusters identified using STRUCTURE was 0.087.

Total number of samples analyzed (n)	Four major Bornean elephant range	Lower Kinabatangan	North Kinabatangan	Central Forest	Tabin Wildlife Reserve
43	Lower Kinabatangan (LK)	0.00			
33	North Kinabatangan (DER)	**0.08**	0.00		
127	Central Forest (CF)	**0.11**	**0.12**	0.00	
21	Tabin Wildlife Reserve (TWR)	**0.11**	**0.14**	**0.03**	0.000

Bold indicates significant values of p value < 0.001.

Bornean elephant have very low genetic diversity. Our results also suggest low diversity in Bornean elephants but substantially higher estimates than that were previously shown (*He* = 0.041, *Ho* = 0.014, Fernando et al., 2003). The average expected heterozygosity (*He*) found in our study was 0.27 (SNPs) and 0.34 (microsatellite) across all populations. However, the microsatellite loci used in our study differ from those used by Fernando et al. (2003) and were polymorphic in the populations on which they were tested. Recent studies point to the fact that cross-specific amplification using highly polymorphic markers provides a biased picture of the genetic diversity when compared with randomly specific markers (Chikhi, 2008; Queirós et al., 2014). Hence, the genetic diversity values obtained in our study are likely overestimated. Such overestimated values are, as expected, in agreement with a previous study by Ahlering et al. (2011), who analyzed elephant populations from Laos using nine microsatellites (same as those used here) and found estimates of genetic diversity (*He* = 0.75) that are much higher than those previously reported (*He* = 0.48) in Fernando et al.'s, 2003 study. Even though these values might be overestimated, levels of genetic variation in Bornean elephants are still similar to or lower than that observed in other rare and endangered species with low levels of genetic variation, e.g. Iberian lynx *He* = 0.31–0.46 (Casas-Marce et al., 2013), European bison *He* = 0.28 (Tokarska et al., 2009), and Ngorongoro Crater lion *He* = 0.58 (Antunes et al., 2008).

Although the Bornean elephants are characterized by low genetic variation at microsatellite loci, we found signature of a recent demographic bottleneck only in the Deramakot and Lower Kinabatangan (but not under SMM) elephant population. If all three mutation models had given the same significant result in each of the population, this would have suggested that the signal was strong enough to be detected using a summary statistics approach.

Interestingly, the pattern of genetic variation inferred by SNP genotypes obtained using elephant fecal and blood samples were also similar. While this would be consistent with a higher diversity in LK in agreement with microsatellite data, the fact that sample sizes differ suggests that further sampling and validation is required.

We found overall concordance among markers (SNPs and microsatellites) in the amount of genetic variation within elephant populations. For instance, the elephants in the Kinabatangan region (i.e. LK and Deramakot) exhibit relatively high levels of genetic variability despite the fragmentation of their habitat. Some contemporary gene flow between elephant populations within the Kinabatangan region may contribute to the maintenance of these high heterozygosity levels. However, the more likely explanations are that fragmentation is recent and/or that effective population size was greater in the recent past. In any case, erosion of heterozygosity should occur relatively quickly through genetic drift and inbreeding if populations are maintained without gene flow (Hartl and Clark, 1989). Hence, continued maintenance of habitat inter-connectivity in the whole Kinabatangan region is very important to retain the maximal amounts of the high genetic diversity seen.

Overall, we found evidence of weak population structure across the distribution range of the Bornean elephant. SNP markers were not powerful enough for assessment of population structure. On the contrary, microsatellite data indicated that at least two elephant sub-populations exist within Sabah. Further, STRUCTURE and TESS analyses performed on the microsatellite data representing only unrelated individuals, produced results similar to $K = 2$. This also indicates that the population structure detected in our samples is not due to the occurrence of related individuals (Anderson and Dunham, 2008).

Our microsatellite data showed that Bornean elephants from the Lower Kinabatangan and North Kinabatangan (Deramakot) ranges are somewhat isolated from the main elephant populations located in the Central Forest and Tabin Wildlife Reserve, as illustrated in the Bayesian clustering methods. Clearly, genetic grouping of these two populations and their higher levels of genetic diversity than other populations is likely reflective of historical connectivity between the two currently disjunct, but geographically proximate locations. Further, the genetic distinctiveness of these populations from the rest of the elephant populations (for both genotype-based and allele frequency based analyses) is most likely attributed to the recent/historical population bottleneck and associated local effects of genetic drift (e.g. fixation and loss of alleles).

These results also revealed that Bornean elephants show genetic differentiation over small geographic distances. Given the absence of obvious barriers to gene flow and high dispersal abilities, Bornean elephant exhibited unexpected population differentiation over relatively small distances. For instance, the observed genetic differentiation among elephant subpopulations in the Kinabatangan region (i.e. LK and DER) is unexpected (F_{ST} ~ 0.08), given the geographical proximity of the sub-populations and the dispersal capabilities of elephants. These sub-populations are separated by approx. 35 km, a distance within the range of elephant movement (Estes et al., 2012). Similarly, our data suggest reduced gene flow between the elephant populations in North Kinabatangan (Deramakot) and Central Forest ranges, which is the largest elephant population in Sabah (Elephant Action Plan, Sabah Wildlife Department, 2012–2016; Estes et al., 2012). These elephant populations are separated by the Kinabatangan River but topographic features such as rivers do not appear to limit elephant movement, however, elephants do not tolerate land-use activities that alter habitats permanently, such as agriculture and human settlement (Zhang et al., 2015; Elephant Action Plan, Sabah Wildlife Department, 2012–2016). Neither of the Bayesian clustering methods recognized the distinctiveness of the forest sites located in Central Forest range, despite significant differentiation by pairwise F_{ST} analysis (range 0.04–0.07). This result is difficult to interpret based on their close geographical proximity (roughly within 50–100 km) and the presence of the largest continuous forest habitat for elephants in this region (Alfred et al., 2010). These levels of genetic differentiation in Bornean elephant populations imply a strong impact of local genetic drift, indicating that the effective population sizes are very small in each forest fragment and that current gene flow among them is likely limited. Habitat fragmentation in Sabah is very recent and cannot fully explain the overall pattern of observed genetic differentiation. Very large parts of the forest in the Kinabatangan region have been logged and converted into oil palm plantations in the last 50 years. Similarly, Tabin forest was connected to Central Sabah before the establishment of the Lahad Datu-Sandakan road that has affected the connectivity between elephant populations in the entire region and identified as significant barrier to elephant dispersal and gene flow (Elephant Action Plan, Sabah Wildlife Department, 2012–2016). However, considering the time lag between the recent landscape changes in Sabah and the beginning of genetic sampling, the Lahad Datu-Sandakan road was probably too recent for a significant barrier. It is known that significant time lags can exist for genetic discontinuities to develop after barrier formation (Landguth et al., 2010) and these time lags make it especially difficult to detect changes in gene flow for species with long generation times such as elephant. As such, some of the observed patterns might be reflective of past rather than present levels of connectivity and fragmentation.

It is also possible that part of the observed pattern of genetic differentiation is influenced by the inclusion of a relatively high percentage of closely related individuals in our sampling scheme. The results from Mantel test demonstrate high genetic relatedness at small spatial scales and suggest that individuals were less genetically related as the geographic distance that separated the two individuals increased. Interestingly, the pairwise relatedness values observed are higher than those reported within family units ($r = 0.37$) for an elephant population in southern India (Vidya and Sukumar, 2005). Our findings concur with the few other studies, indicating how isolation by distance within elephant populations may occur at fine spatiotemporal scales and in the absence of obvious landscape barriers, and may be driven by mechanisms, such as dispersal behavior and species' mating system (e.g. Vidya et al., 2005b). Nonetheless, understanding and modeling

the social organization of Bornean elephants should provide a clearer picture of the current distribution of genetic diversity and population structure (e.g. Parreira and Chikhi, 2015).

4.2. Null alleles and genotyping error

Using microsatellite markers, we observed a statistically positive F_{IS} for all Bornean elephant populations studied herein, indicating a deviation from HWE. The most likely explanations include the presence of null alleles and non-random mating. Additionally, a Wahlund effect (due to sampling several genetically differentiated sub-populations) should not be ruled out as contributors to the deviation from HWE. Many individuals also exhibited high relatedness values with members of the family units ($r > 0.34$). This may explain the deviation of HWE for the two populations (DER and CF) after the genotypes were corrected for null alleles.

The lower rate of SNP genotyping success observed in this study is most likely due to the low DNA concentration of fecal samples and high multiplexing levels. One limitation of MALDI-TOF mass spectrometer analysis, as implemented in Sequenom, is the purity of the sample required by the assay (Sobrino et al., 2005). Fecal DNA normally includes other sources of DNA from diet and is always more degraded than blood or other tissue types (Taberlet et al., 1996). Indeed, an error frequently identified in our data set was the inaccurate calling of individual genotypes with individual genotypes fell between the three main genotype clusters. In this case, the alleles were treated as missing data. Further, MassARRAY assays in Sequenom employ a classical PCR technology that requires multiple sets of primers to amplify multiple specific regions. Such specificity requirement becomes even more critical with increasing level of multiplexing.

The positive correlation between SNP genotype error rate and multiplexing level suggests that genotyping performance rate (accuracy) of SNPs from fecal samples decreased with increasing multiplexing level. Multiplex PCR is a sensitive technique and the methods for multiplexing markers, such as microsatellites have considerably improved over the last years due to the use of specialized PCR protocols, such as optimized PCR buffers, the Qiagen multiplex PCR kit, and Touch-down PCR (Guichoux et al., 2011). However, for highly multiplexed sets of SNPs (>28), more advanced strategies might still be necessary and additional efforts to improve SNP multiplex genotyping and scoring remain critical. Unfortunately, genotyping error rates are not available for Sequenom assays in other non-model species. However, comparable values are available from a recent study that has tested the efficiency of SNP genotyping from fecal DNA of Italian wolf using a different genotyping assay (TaqMan) (Fabbri et al., 2012). This study found a high percentage of positive PCRs (86–92%) and low ADO rate (0–18%).

Our study suggests that the polymorphic SNPs were useful for estimating general level of genetic diversity in Bornean elephants but closely related populations were generally better distinguished with microsatellites than SNPs. Panels of SNPs are rapidly becoming the population genomic markers in ecological and conservation genetics studies (Morin et al., 2004; Kraus et al., 2015), however, given concerns regarding error rates and monomorphic SNP calls, these panels have only limited value, especially when working with genetically depauperate populations, as seen in this study. Simulated (Morin et al., 2009) and empirical data (Tokarska et al., 2009) have shown that significant power (>0.95) to detect population structure at very recent divergence times required as many as 15 times more SNPs than microsatellites (Haasl and Payseur, 2011). The present study includes many fewer SNP loci and we will still need to apply more loci for a finer resolution of population and conservation genetics of Bornean elephants in future studies. Also the choice between the genetic markers will be best determined by the questions being tested, as well as laboratory specific costs and technical capabilities (McMahon et al., 2014).

4.3. Conservation implications

This study gives the first description of the genetic diversity and structure of Bornean elephant populations across Sabah (i.e. most of the species range) and as such should be used for their conservation management. The failure to identify variation in the mtDNA control region of Bornean elephants, in combination with the microsatellite and SNP data, lends support to the conclusion that Bornean elephants exhibit overall low genetic variability. The detection of reduced gene flow levels among elephant populations in Sabah adds to a growing body of literature that documents an increasingly fragmented landscape for large mammals in Borneo. One of the most important management-related results from this study is that significant genetic differentiation exists between extant elephant populations. Reinforcing gene flow by re-establishing habitat connectivity between populations, especially between ranges such as the Kinabatangan, Tabin and Central Forest, may therefore be the priority given that the Kinabatangan elephant population appears to be the most genetically diverse. Our study also reveals that genetic diversity is unequally distributed between the elephant populations in Sabah that are often small and isolated. If populations decrease too much in size, they will become more sensitive to stochastic events. Inbreeding and loss of genetic variation are inevitable consequences of small population sizes (Saccheri et al., 1998; Frankham, 2010). It is not known how long the elephant populations have been small but it could be that in populations that experience small sizes may be less susceptible to future inbreeding depression because they have been purged of deleterious recessive alleles. Also, genetic diversity can be restored with introduction of individuals from other mainland elephant population, however, it also carries the risk of outbreeding depression and needs careful evaluation (Frankham et al., 2011). Nevertheless, the elephant populations in Borneo may require more intensive genetic management and the possible expression of inbreeding depression should be carefully followed.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.biocon.2016.02.008.

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