Using fecal-DNA and capture-mark-recapture to establish a baseline Asian elephant population for the Eastern Plains Landscape, Cambodia

Thomas N.E. Gray[1], T.N.C. Vidya[2], Andrew L. Maxwell[1], D.K. Bharti[2], Sheetal Potdar[2], Phan Channa[1] and Prum Sovanna[1]

October 2011

WWF Greater Mekong Cambodia Country Program 1

Jawaharlal Nehru Centre for Advanced Scientific Research 2

Abstract

Protected areas of the Eastern Plains Landscape, eastern Cambodia have been identified as critical for the conservation of wild Asian elephant *Elephas maximus* in Indochina. A 2006 study in Seima Protected Forest, using fecal-DNA samples and capture-mark-recapture analysis, estimated an Asian elephant population of between 101 and 139 individuals. We conducted a similar fecal-DNA based capture-mark-recapture study in order to establish a monitoring baseline for Asian elephant in two additional protected areas, Phnom Prich Wildlife Sanctuary (PPWS) and Mondulkiri Protected Forest (MPF), in the Eastern Plains Landscape. Five field sampling sessions between February and May 2009 collected 270 fecal samples from the core areas of PPWS (226 samples) and MPF (44 samples). Samples were sent to the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India for genotyping. Amplification rates varied between survey teams and sampling sessions but overall were 68%. A total of 98 wild Asian elephant individuals were identified; 78 in PPWS and 21 in MPF. Using model-averaging of biologically plausible closed capture-mark-recapture models in software MARK the Asian elephant population in PPWS was estimated at 136 ± SE 18 individuals. Using continuous-occasion capture-mark-recapture models in software CAPWIRE the PPWS population was estimated at between 101 and 175 (mean estimate 154) individuals. No individual Asian elephants were recaptured between sampling sessions within MPF and thus the number of individuals recorded, 21, must be regarded as a minimum population estimate for the protected area. Recaptures of elephants within PPWS provided evidence of movements within the protected area particularly in, and around, the Kranchilok semi-evergreen forest block. This area appears to support the majority of the PPWS Asian elephant population during the dry-season and requires the highest level of protection. One individual Asian elephant was recorded in both PPWS and MPF, suggesting that at least some elephants use both protected areas, thus highlighting that landscape scale management across protected area boundaries is essential for the persistence of a viable Asian elephant population in the landscape. GPS or satellite collaring is recommended to further understand Asian elephant movements across the Eastern Plains Landscape. As part of the WWF Greater Mekong 2011-2015 strategic plan Asian elephant population monitoring, using the same methodologies as in this study, will be repeated every 3-4 years.

Khmer Abstract

តំបន់អភិរក្សទេសភាពខ្ពង់រាបភាគខាងកើតនៃប្រទេសកម្ពុជា ត្រូវបានទទួលស្គាល់ថាជាតំបន់ដែលមានសារសំខាន់ខ្ពស់សំរាប់ការអភិរក្សសត្វដំរីព្រៃអាស៊ី។ ការសិក្សាស្រាវជ្រាវដែលធ្វើឡើងនៅក្នុងតំបន់អភិរក្សជើរចិះកែវសីមាឆ្នាំ២០០៦ ដោយប្រើវិធីសាស្ត្រវិភាគសំណាក DNA ដីរនិងការវិភាគ capture-mark-recapture បានធ្វើការប៉ាន់ប្រមាណសត្វដំរីព្រៃថាមានចំនួនប្រមាណពី ១០១ ទៅ ១៣៩ក្បាល។យើងបានអនុវត្តវិធីសាស្ត្រដូចគ្នាខាងលើនេះដើម្បីធ្វើការប៉ាន់ស្មានចំនួនសត្វដំរីធ្វើជាមូលដ្ឋានក្នុងការតាមដានបន្ថែមទៅលើតំបន់អភិរក្សទាំងពីរគឺដែនជីរកសត្វព្រៃភ្នំព្រេចនិងព្រៃការពារមណ្ឌលគីរីនៃតំបន់ទេសភាពខ្ពង់រាបភាគខាងកើតនៃប្រទេសកម្ពុជា។ ការប្រមូលសំណាកត្រូវបានធ្វើ៥លើកជាប់ៗគ្នាគឺចាប់ផ្តើមពីខែកុម្ភៈ ដល់ខែឧសភា ឆ្នាំ២០០៩ ហើយប្រមូលបាន២៧០សំណាកដែលក្នុងនោះតំបន់ស្នូលក្នុងដែនជីរកសត្វព្រៃភ្នំព្រេចទទួលបាន២២៦សំណាក និងតំបន់ព្រៃការពារមណ្ឌលគីរី៤៤សំណាក។ សំណាកទាំងអស់ត្រូវបានបញ្ជូនទៅវិភាគនៅមជ្ឈមណ្ឌល Jawaharlal Nehru នៃវិទ្យាស្ថានស្រាវជ្រាវវិទ្យាសាស្ត្រជាន់ខ្ពស់បឹងហ្គាឡូ ប្រទេសឥណ្ឌា។ ការប្រមូលសំណាកមានភាពខុសគ្នារវាងក្រុមមួយទៅក្រុមមួយ និងពីមួយលើកទៅមួយលើកតែជាទូទៅអត្រាវិភាគដែលសំរេចបានមានប្រមាណ៦៨%។ ចំនួនសត្វដំរីព្រៃសរុប៩៨ក្បាលត្រូវបានធ្វើអត្តសញ្ញាណដោយការវិភាគ DNA ហើយក្នុងនោះមាន៧៨ក្បាល មាននៅឧទ្យានជាតិភ្នំព្រេច និង២១ក្បាលនៅតំបន់ព្រៃការពារមណ្ឌលគីរី។ ការប្រើប្រាស់ម៉ូដែលនិងកម្មវិធី Mark បានធ្វើការប៉ាន់ស្មានសត្វដំរីព្រៃសរុបមាននៅក្នុងដែនជីរកសត្វព្រៃភ្នំព្រេចគឺមានប្រហែលពី១១៨ ទៅ ១៥៤ ក្បាល។ ដោយការប្រើកម្មវិធី CATWIRE ចំនួនសត្វដំរីព្រៃដែលប៉ាន់ស្មានមាននៅក្នុងដែនជីរកសត្វព្រៃភ្នំព្រេចមានចំនួនពី ១០១ ទៅ ១៧៥ ក្បាល (មានប្រហែល ១៥៤ក្បាលតាមការគណនាមធ្យម)។ គ្មានចំនួនសត្វដំរីព្រៃត្រូវបានចាប់រាប់ឡើងវិញក្នុងពេលសិក្សានៅតំបន់ព្រៃការពារមណ្ឌលគីរី ដូចនេះដំរីព្រៃចំនួន ២១ក្បាល ដែលបានគណនារកឃើញគឺជាចំនួនអតិបរិមាដែលបានធ្វើការប៉ាន់ស្មាននៅក្នុងតំបន់នេះ។ ការចាប់រាប់សារឡើងវិញនៃសត្វដំរីនៅក្នុងដែនជីរកសត្វព្រៃភ្នំព្រេចបានផ្តល់ភស្តុតាងដ៏សំខាន់នៃការធ្វើបំលាស់ទីនៅក្នុងតំបន់ការពារ ជាពិសេសនៅក្នុងនិងជុំវិញតំបន់ក្រង់ជ្រលក់នៃតំបន់ព្រៃពាក់កណ្តាលស្រោង។ តំបន់នេះមានទាំគ្រៃយ៉ាងសំខាន់ដល់ចំនួនសត្វដំរីព្រៃភាគច្រើននៅក្នុងអំឡុងរដូវប្រាំងហើយទាមទារឱ្យមានការការពារមានកិរិតខ្ពស់។ មានសត្វដំរីព្រៃមួយក្បាលបានធ្វើបំលាស់ទីរវាងតំបន់ដែនជីរកសត្វព្រៃភ្នំព្រេចនិងតំបន់ព្រៃការពារមណ្ឌលគីរី នេះបានគូសបញ្ជាក់ប្រាប់ថាការគ្រប់គ្រង់តំបន់ទេសភាពដោយឆ្លងព្រំប្រទល់តំបន់ការពារទាំងពីរ មានសារសំខាន់សំរាប់ភាពស្ថិតស្ថេរនៃមុខងារ (មុខងារស្ថិតិសត្វដំរីនៅក្នុងតំបន់ទេសភាព)។ ការបំពាក់ឧបករណ៍តាមដានបំលាស់ទីផ្កាយ រណប ឬ GPS គឺជាអនុសាសន៍សំរាប់ការសិក្សាស្រាវជ្រាវដើម្បីស្វែងយល់អំពីការបំលាស់ទីរបស់សត្វដំរីពាស់ពេញតំបន់ទេសភាពសំរាប់ ពេលអនាគត។ ការតាមដាននិងត្រួតពិនិត្យសត្វដំរី ជាផ្នែកមួយនៃផែនការយុទ្ធសាស្ត្ររបស់កម្មវិធីអភិរក្សអង្គការ WWF មហាតំបន់ទន្លេមេគង្គ ២០១១-២០១៥ ដោយប្រើវិធីសាស្ត្រដូចគ្នាក្នុងការសិក្សាស្រាវជ្រាវដែលនឹងត្រូវរៀបចំឡើងរៀងរាល់ ៣-៤ ឆ្នាំម្តង។

Introduction

Asian elephant, *Elephas maximus*, are globally endangered and restricted to isolated populations across their ancestral range, where they are threatened by poaching, illegal capture for trade, habitat loss and fragmentation, and human-elephant conflict (Leimgruber *et al.* 2003, Choudhury *et al.* 2008, Loucks *et al.* 2009, Webber *et al.* 2011). Despite the Asian elephants' perceived status as a conservation flagship, there are few robust population estimates from the species' global range. Blake and Hedges (2004) argued that the oft-repeated global population 'estimate' of 40,000 to 50,000 individuals is no more than a crude guess and that a lack of statistical rigor in survey design, the use of nonstandard methods, and the belief that guesses are sufficient for the purposes of conservation planning have hindered Asian elephant conservation efforts. Despite increased appreciation in the conservation community of the importance of robust monitoring and the use of evidence for conservation decision making (Sutherland *et al.* 2003, Nichols & Williams 2006, Pullin & Knight 2009) there remain few robust Asian elephant population estimates from anywhere within the species' range . In South-east Asia robust Asian elephant population estimates, accounting for the most problematic aspects of animal abundance estimation, spatial sampling, and detectability (Williams *et al.* 2002), are available for only four populations (Bukit Barisan Selatan and Way Kambas National Parks, Sumatra; Hedges *et al.* 2005; the Nakai Plataeau, Lao PDR; Hedges *et al.* 2007; and Seima Protected Forest, Cambodia; Pollard *et al.* 2008).

Effective surveys of Asian elephant in South-east Asia are hindered as the species is generally shy and elusive and occurs at low densities in often inaccessible and densely forested habitat. However, the species' abundant, and easily identifiable, dung provides opportunities for survey. Line-transect based dung counts, with appropriate corrections for defecation and decay rates, have been used to estimate Asian elephant population size in Sumatra (Hedges *et al.* 2005). However doubts remain about the suitability of this approach when elephant density is low and the area of interest is large (Hedges & Tyson 2002, Hedges & Lawson 2006). Reliable estimation

of dung decay rates is also prohibitively difficult when only a few fresh dung piles can be located (Barnes 2002, Laing *et al*. 2003, Hedges & Lawson 2006).

The use of non-invasive genetic sampling, in which unique genotypes are extracted from DNA, to estimate abundance has been used for a variety of taxa including tigers *Panthera tigris* in India (Mondol *et al.* 2009), wolves *Canis lupus* in Europe (Cubaynes et al. 2010), bears *Ursus* spp. in Europe (Bellemain *et al.* 2005) and North America (Boulanger *et al.* 2008), primates (Arandjelovic *et al*. 2010) and African elephant *Loxodonta africana* (Eggert *et al.* 2003) in Africa, and a variety of bird species (Rudnick *et al.* 2005, Jacob *et al*. 2010). Such analysis can be based on fecal samples (Eggert *et al.* 2003, Mondol *et al.* 2009) or other tissue such as hair (Mulders *et al.* 2007, Gardner *et al*. 2010) or feathers (Rudnick *et al.* 2005). Population size is usually estimated within a capture-mark-recapture statistical framework (Williams *et al.* 2002). Capture-mark-recapture on Asian elephant fecal-DNA samples has successfully been used to estimate population size in Lao PDR (Hedges *et al.* 2007) and Cambodia (Pollard *et al*. 2008) – the only robust Asian elephant population estimates from mainland South-east Asia. However there are no peer-reviewed publications using robust capture-mark-recapture analysis on fecal DNA to estimate elephant population size from Asia, and only one from Africa (Eggert *et al.* 2007), although a number of papers have used DNA genotyping to obtain minimum population sizes (Eggert *et al.* 2003, Vidya *et al.* 2007, Ahlering *et al.* 2011).

The status of Asian elephant in Cambodia is unclear and the regularly quoted population estimate of between 250 and 600 individuals (Sukumar 2003, Choudhury *et al.* 2008, Webber *et al.* 2011) is only an educated guess. Substantial Asian elephant populations in Cambodia appear restricted to the mountains of south-west Cambodia, and evergreen and semi-evergreen forest in southern and eastern Mondulkiri province. A smaller population occurs in Preah Vihear province along the Laos border whilst relict, and small, Asian elephant populations may remain in, and around, Virachey National Park (Rattanakiri/Stung Treng), Kirioum National Park (Kompong Speu) and Prey Long (Stung Treng/Kratie). A 2006 study in south and east Seima Protected Forest, Mondulkiri, based on capture-mark-recapture analysis of fecal-DNA, estimated an Asian

elephant population of between 101 and 139 individuals (Pollard *et al.* 2008). A similar study covering the entire Cardoman mountains forest complex of south-west Cambodia identified approximately 175 individual elephants but no individuals were re-captured making capture-mark-recapture analysis impossible (M. Maltby/FFI *in litt*.).

We report on an Asian elephant fecal DNA study from Phnom Prich Wildlife Sanctuary and Mondulkiri Protected Forest, two adjacent protected areas in eastern Cambodia. We use a capture-mark-recapture statistical framework to estimate the Asian elephant population at these sites in order to provide a robust baseline population estimate.

Methods

Study Area

Phnom Prich Wildlife Sanctuary (c. 2,250-km^2 and centered on 12.70^{o}N; 106.80^{o}E) and Mondulkiri Protected Forest (c. 3,300-km^2 and centered on 13.00^{o}N; 107.35^{o}E) form part of the Eastern Plains Landscape, one of the largest protected area complexes in tropical Asia, which also includes Seima Protected Forest, Lumphat Wildlife Sanctuary and Yok Don National Park, Vietnam (Fig. 1). Both PPWS and MPF are dominated by deciduous dipterocarp forest with smaller patches of semi-evergreen and mixed deciduous forest at slightly higher elevations and along water courses. Biodiversity surveys have provided evidence that the Eastern Plains Landscape is globally or regionally significant for the conservation of Asian elephants (Pollard *et al.* 2008), primates (Pollard *et al.* 2007; Rawson *et al.* 2009; Phan & Gray 2010-a), large ungulates (Phan & Gray 2010-b; O'Kelly & Nut 2010, Gray *et al.* 2011), leopard *Panthera pardus* and other carnivores (Gray *et al.* 2010, Gray & Prum 2011; Gray *et al.* submitted), and large waterbirds and vultures (Seng *et al.* 2003; Wright *et al* in press; Gray *et al.* in prep).

Despite a number of unconfirmed reports of large numbers of Asian elephant in what is now MPF, preliminary surveys in the late 1990s found few wild elephant signs (Desai & Lic 1996; Duckworth and Hedges 1998; Long *et al.* 2000). With no contemporary data available from Phnom Prich Wildlife Sanctuary, Duckworth and Hedges (1998) concluded that '*large numbers'* of Asian elephant '*do not persist widely in Ratanakiri and Mondulkiri'* with the possible exception of Keo Seima district [south of PPWS] which '*seemed to have important numbers'*. However, following the surveys conducted between 2000 and 2001, PPWS was recognized as a core area for the potential elephant meta-population ranging over northeastern Cambodia and southern Lao PDR (Timmins & Ou 2001, Desai *et al.* 2002). Between 2001 and 2007 more than 80 independent camera-trap photographs of Asian elephant were obtained from PPWS with up to 10 individuals per photographs and an estimate of a minimum of 42 individual elephants at three closely clustered sites (WWF-internal data).

Camera-trapping and field surveys since 2005 have produced fewer Asian elephant records from MPF. However there is some evidence of wet-season dispersal east into the protected area from PPWS with observations of at least 22 elephants crossing the main road between the protected areas annual since 2006 (WWF-internal data). Year-round records from a small area in the east of MPF, south of the Srepok river and adjacent to the Vietnam border, suggest a small resident population which may also use Yok Don national park, Vietnam (WWF-internal data).

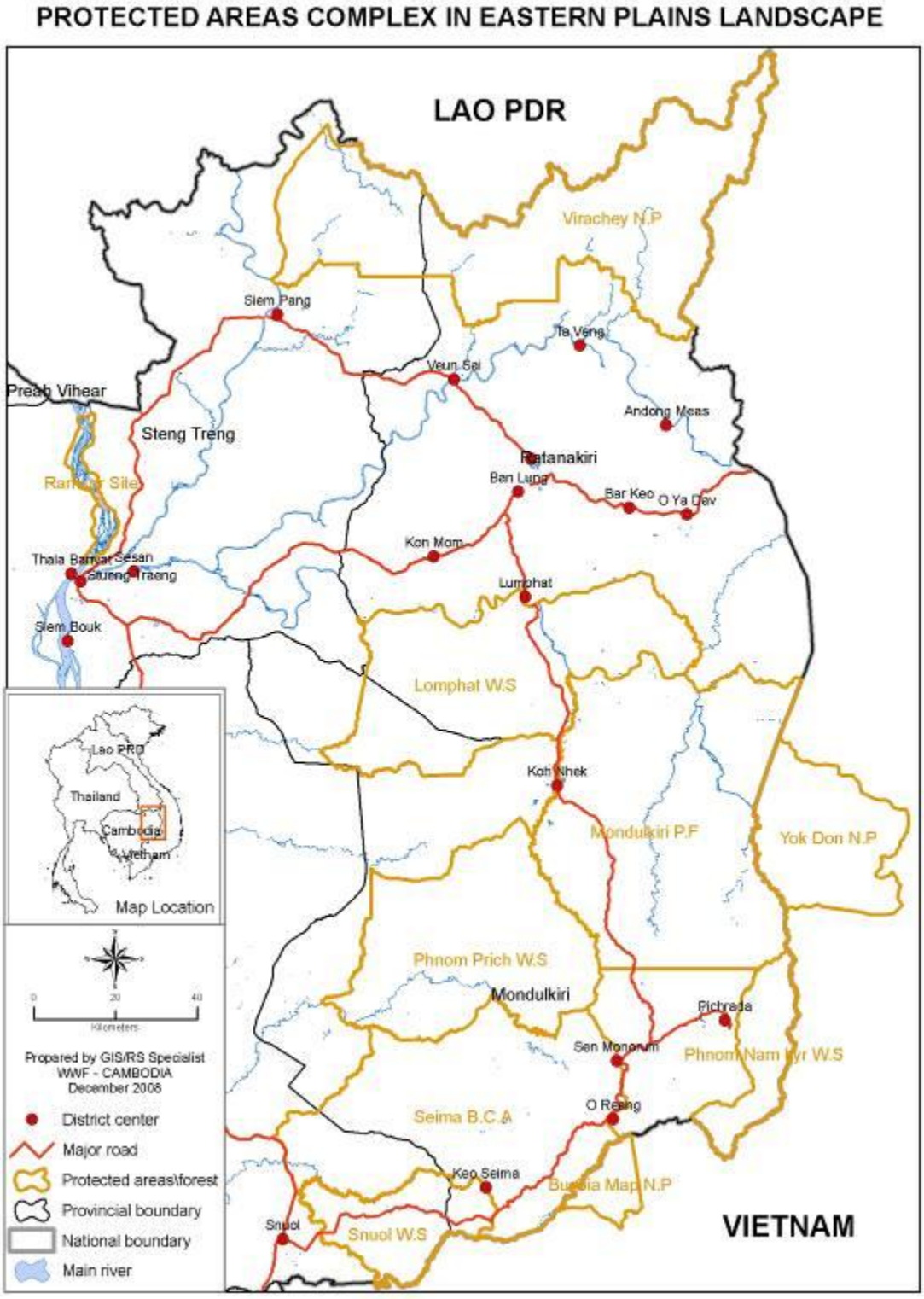

Figure 1. Protected Areas within the Eastern Plains Landscape Cambodia and Vietnam

Field sampling design and survey methodology

Sampling design followed the principles of closed capture-mark-recapture studies (e.g. as described for tiger using camera-traps by Nichols & Karanth (2002)) and was also guided by the MIKE protocols for estimating elephant population densities using fecal DNA-based capture–recapture sampling (Hedges & Lawson 2006). Elephant fecal-DNA samples were collected during five sampling sessions. These were used as primary samples during capture-mark-recapture analysis. Sampling was designed to ensure approximately equal survey coverage across all key dry-season Asian elephant locations during each sampling session thereby ensuring all individuals had non-zero probabilities of being sampled. To maximise the chances of demographic and geographic closure across the study period (i.e. that the Asian elephant population in the study area did not change due to birth, death, immigration, or emigration) the study was conducted during the early 2009 dry-season when elephant movements are restricted due to limited water availability. The overall survey period (<90-days) was also kept to a minimum in order to satisfy requirements of demographic closure.

Sampling was targeted at areas with high probabilities of detecting Asian elephant sign. Therefore camera-trap and patrol data (observations and signs from MIST database) were combined to produce a database of point locations from where wild Asian elephant were recorded between 2001 and 2006 across the core areas of Phnom Prich Wildlife Sanctuary (approximately 1,500-km^2) and Mondulkiri Protected Forest (approximately 1,700-km^2). This information was supplemented with ranger and local villager knowledge to identify Asian elephant hot-spots (approximately 10-15-km^2), where high concentrations of elephant sign had been recorded, and additional survey blocks (30-60-km^2), where elephants were known or believed to occur. A total of eleven hot-spots and four survey blocks were identified in PPWS with four hot-spots and three survey blocks in MPF (Fig. 2).

Sampling was conducted between 3/2/2009 and 3/5/2009 with each sampling session lasting between 8 and 10 (mean 8.2) days with between 10 and 13 (mean 12) days between successive sampling sessions:

1. Sampling session one: 3-10/Feb/2009 (7 days)
2. Sampling session two: 23/Feb-3/Mar/2009 (8 days)
3. Sampling session three: 15-23/Mar/2009 (8 days)
4. Sampling session four: 2-12/Apr/2009 (10 days)
5. Sampling session five: 25/Apr-3/May/2009 (8 days)

Four two-man survey teams were each allocated between 3-4 hot-spots and 2-3 survey blocks (Fig. 2). Prior to, and during, the first survey session all survey teams were trained by Prum S., A. Maxwell and T. Gray on MIKE protocols for sterile collection of dung samples. During each sampling session each survey team visited all hot-spots and survey blocks in their allocated area searching for elephant sign. Survey teams focused searches around deep water pools in otherwise dry river channels, natural springs, salt-licks, animal tracks moving through the semi-evergreen forest, and the eco-tone between the semi-evergreen and deciduous dipterocarp forest. Survey teams also followed up all local reports of recent elephant activity including areas away from previously designated hot-spots and survey blocks.

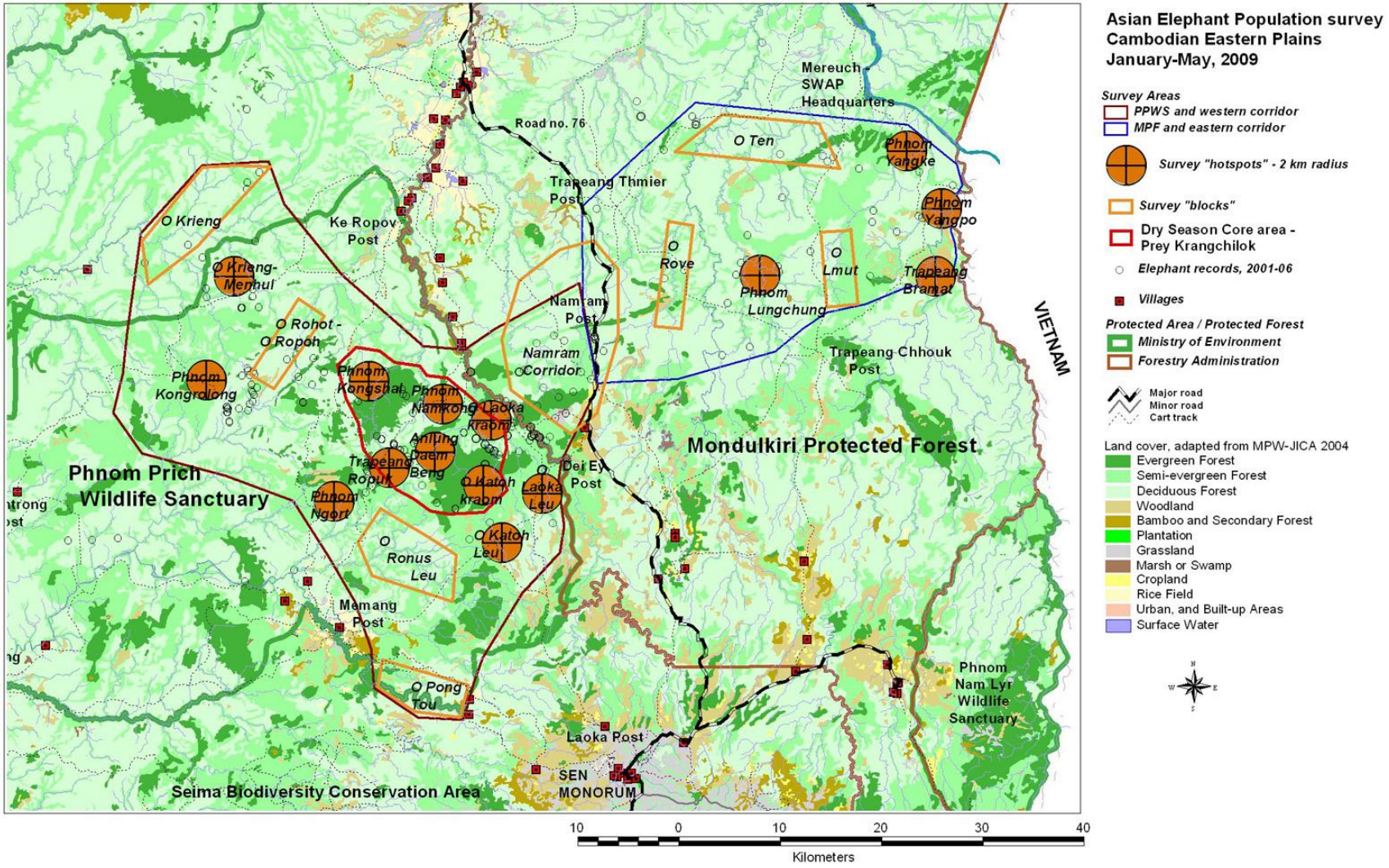

Figure 2. Asian elephant hot-spots and survey blocks in PPWS and MPF

Upon encountering elephant sign survey teams collected samples from all fresh and reasonably fresh dung piles aged using MIKE criteria (Hedges & Lawson 2006). When multiple dung piles were associated with a group of elephants efforts were made to collect samples from all different individuals present, based on bolus size and distribution of dung piles, provided they were classified as fresh or reasonably fresh. All samples were collected in a standardised way that minimised the chance of cross contamination and only dung from the outer coating of each bolus was collected. For each collected sample approximately 10 grams of dung were places into 40-ml polypropylene tubes filled with approximately 20-ml of Queen's College Buffer (20% DMSO, 100 mM Tris pH 7.5, 0.25 M EDTA, saturated with NaCl; Amos *et al.* 1992). After collecting dung samples the maximum circumference of up to three intact boli in the dung-pile was measured using a plastic measuring tape. If more than three boli were present, the largest three were measured.

Between the 13/June/2009 and 9/July/2009 samples were also collected from all domestic elephants known to use the study area using the same collecting protocol as for wild elephant. All samples were transported to Phnom Penh where they were refrigerated and were subsequently air-freighted to the Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India for genetic analysis.

Genetic analysis and laboratory methodologies

DNA Extraction

For each of the fecal-DNA samples, approximately 0.25 ml of the dung was transferred into a 2.0 ml microcentrifuge tube and about 1.0 ml of digestion buffer (100mM NaCl, 10mM Tris pH 8.0, 25mM EDTA pH 8.0, 2% SDS) was added, mixing the dung into the digestion buffer thoroughly. To this, 20 µl of Qiagen Proteinase K (20 mg/ml) was added and the samples incubated overnight at 55ºC in a shaker-incubator. After the digestion, the samples (digests) were vortexed and then centrifuged at 13,000 rpm for 2 minutes. 400 µl of the supernatant was transferred to a fresh 2.0 ml microcentrifuge tube and the remaining digest was stored at -20ºC for a possible second extraction if required. To the supernatant, 800 µl of Phenol-Chloroform-IsoAmyl Alcohol (25:24:1, Amresco Inc.) was added, mixed, and incubated at 55ºC for about 45 min. The sample was vortexed and then centrifuged at 13,000 rpm for 2 minutes again. The aqueous layer, which is expected to contain DNA, was pipetted out carefully and transferred to a fresh 1.5 ml microcentrifuge tube. This extract was then purified using silica columns and buffers provided as part of the QIAquick PCR purification kit (Qiagen) and following the manufacturer's protocol. The purified DNA was eluted into 50 µl of elution buffer and stored at -20ºC. To check if the DNA yield was sufficient for PCR (Polymerase Chain Reaction) amplification, a test reaction was carried out using the primers EMU04 and FH94 with seven of the extracted samples, all of which showed positive amplification of at least one of the loci.

Since the initial test amplifications were good, some extractions were carried out using the method explained above. However, it was realized later that many samples did not amplify. This could have been either due to the presence of inhibitors in the DNA extract, which would require smaller volumes of extract to be used, or due to too little DNA present in the extract, which would require larger volumes or more concentrated extracts to be used. It is possible to discriminate between the two possibilities by carrying out PCRs with different volumes of DNA extract and finding out if samples that amplified initially stopped showing amplification when more DNA extract volume was used (PCR inhibition) or if samples that did not work initially showed amplification when more DNA was used (too little DNA). It was found that inhibitors

were not so much the problem as too little DNA in the extracts. Therefore, during subsequent extractions, purified DNA was eluted into 30-40 µl, instead of 50 µl, of elution buffer in order to concentrate the DNA. Since many samples did not show PCR amplification, re-extractions were carried out as the DNA extracts were insufficient to repeat PCRs. In all, 638 extractions were done for the 298 samples.

PCR amplification of microsatellites:

DNA extracts were amplified at 10 previously published microsatellite loci (Table 1). PCR reactions were carried out in 12.5 µl volumes, consisting of 9.0 µl of PCR mix (8988.44 µl water, 19.6 µl 1M MgCl2, 130.9 µl 1M Tris [pH 8.4], 163.66 µl 4M KCl, 131 µl of each of 4 dNTPs, 173.4 µl 1000mg/ml BSA for 10ml of master mix), 0.25 µl of each 10 µM primer, 0.1 µl of 5 U/µl Taq DNA polymerase (Fermentas Life Sciences, Thermo Fisher Scientific Inc.), 2-2.9 µl DNA, and the remaining volume, if any, made up with water. A 1:2 dilution of EMU10 primers was used, as it helped improve allele peak clarity in the electropherogram. Multiplexing was possible only for EMU04 and FH94, and EMU10 and EMU14 because of their product sizes, fluorescent labels and annealing temperature compatibility (Table 1). For the multiplex PCRs, 0.25 µl of forward and reverse primers for each locus were added. The PCR consisted of an initial denaturation step at 95ºC for 3 minutes, followed by 40-45 cycles of three steps. These three steps included denaturation at 93ºC for 1 minute, followed by annealing for 1 minute, and extension at 72ºC for 1 minute. In the case of some loci, a 'touchdown' approach was taken, in which the annealing temperature was reduced by 0.5ºC every cycle from cycle 11-31, so as to increase amplification. The three-step cycles were followed by a final extension at 72ºC for 15 minutes, and the products were then stored at 4ºC. The annealing temperatures were standardized afresh for the samples in this study to eliminate the presence of non-specific bands which were observed with these samples. The difference in annealing temperatures used by us from those published are likely to be largely due to differences in lab conditions (PCR machine ramping speed, reagents etc). However, there were also (often small) differences between annealing temperatures that had been already standardized for the same loci while using fresh dung samples from southern India for another study. Since the lab conditions in this case were the same, the differences may have resulted from differences in sample quality, because of 1)

inherent differences in sample quality at the time of collection because of the age of the dung or because of different inhibitors present in the dung, resulting from the elephants being in different habitats, or 2) differences in quality of extract because of how samples were stored (the southern Indian samples were stored in ethanol). The standardized annealing temperatures which were eventually used in this study are shown in Table 1. Every set of PCR reactions included a negative control in which the DNA extract was replaced by water. Several sets of PCR reactions also included positive controls, which were DNA extracts from fresh dung samples (obtained within a couple of hours after observed defecation) from southern India, which we had previously amplified successfully. Great care was taken to prevent cross contamination of samples while carrying out DNA extractions and PCR amplifications. Separate areas and instruments were always used for handling pre- and post-PCR products. Filter tips were always used, which contain barriers against aerosol dispersal - a potential source of contamination during pipetting. Since dung is a poor source of DNA, every sample was initially PCR amplified twice at each locus

Locus	**Repeat unit**	**Primer sequence (5' - 3')**	**Label**	**T_a**	**T_a***	**Allele range (in bp)**
EMU03	$(GT)_6GC(GT)_8$	F: AGAAGCAAAACCCATGAAGC R: TTGAAACTTGCCAGCCTCTT	PET*	58	64	137-143
EMU04	$(TG)_{12}$	F: TGACTCTCCCTCTTCTGCATC R: GGCTGAGAGGGAAAGAAATTG	6-FAM	58	65	97-107
EMU07	$(TG)_{15}$	F: GAGCAGTGCCTTTCGTGAC R: AGCCTGGGAGGTAAGTAGCA	6-FAM	58	68	100-124
EMU10	$(CA)_{17}$	F: AATCGACTCAGCAGCAACAG R: CCAGTAAATCCATATCACTCGTC	PET	58	64	94-104
EMU12	$(AC)_8$	F: CCAAAGAAGACCCATGTTCC R: CTGACTATGGGGGAGACTGC	VIC	58	63	120-152
EMU13	$(GT)_{17}$	F: GTATTTGGGCTGGCATGGT R: GTGGGGTCTGTGGTCAAGTG	PET	58	55	100-110
EMU14	$(GT)_{15}$	F: GCCTACATGCAGGGTTTGC	6-FAM	58	64	130-140

		R: TGAGCCTCTGGCATTTATGA				
EMU15	$(AC)_{14}$	F: TTCGGGATGTTCTCTTCTGT	VIC	58	60	142-154
		R: GGGGCTTAACTAATAGGCTTCA				
EMU17	$(GT)_{16}$	F: CACTCAGAGTTCCAAGAAGCAG	PET	58	64	119-137
		R: TGCCAGCCATTTCCTCTC				
FH94	$(CA)_{16}$	F: TTCCTCCCACAGAGCAGC	NED	61	65	229
		R: ATTGGTTAATTTGCCAGTCCC				

* The label NED was used in this study instead of PET.

Table 1. Details of microsatellite loci used in this study, taken from Kongrit et al. (2007) and Comstock et al. (2000). T_a is the annealing temperature provided in the initial paper, while T_a^* is the annealing temperature we used after standardizing. The allelic ranges are also from the original papers.

If both these PCRs produced the same heterozygote, no further PCRs were carried out for that sample at that locus. If both the PCRs produced the same homozygote, we looked at the intensity of the peak in the electropherogram to decide whether to re-run the sample a third time. This was done for many samples since the peaks were not of high intensity. If the two PCRs did not work or if they showed different results, they were rerun up to an additional six times, making it a total of eight PCRs for that sample at that locus (this was, however, rare). If a sample did not work at most or all of the loci after trying each PCR twice, it was not re-run again more than once because the probability of it amplifying was very low.

In all, 9289 PCRs were carried out excluding the negative and positive controls (an additional few hundred PCRs). The breakup according to locus is as follows: EMU12 – 1006, EMU04 – 869, FH94 – 1025, EMU10 – 820, EMU14 – 829, EMU07 – 869, EMU15 – 887, EMU17 – 990, EMU03 – 855, EMU13 – 1139. These numbers of PCRs do not necessarily reflect how sensitive each locus was because the loci were amplified in this order given above and, therefore, once several loci were done, we had an idea of the quality of each sample, and poor quality samples were not amplified at the remaining loci many additional times. The last locus, EMU13, was tried repeatedly because even most of the good quality samples did not show amplification at this

locus. Since it had been possible to standardize this locus for samples from southern India in another ongoing study and the same primer stocks were tried, it is possible that there were null alleles at this locus (a situation in which mutations in the primer-binding site preclude amplification) in the Cambodian population. This locus was, therefore, abandoned in further analyses.

Genotyping:

Based on the fluorescent label that each primer was labeled with and the allele range associated with each locus, PCR products of compatible loci were mixed together. This led to four panels: 1) EMU04, FH94, and EMU12, 2) EMU10 and EMU14, 3) EMU07, EMU15, and EMU17, and 4) EMU03 and EMU13. While such mixing of these loci was possible during the first sets of electrophoresis, repeat reactions had to often be electrophoresed as single loci without mixing. PCR products were electrophoresed, along with the internal size standard GS500LIZ, in an ABI PRISM 3730 DNA Analyzer (Applied Biosystems) in the JNCASR Sequencing Facility. Genotypes were scored using the GeneMapper software version 4.0 (Applied Biosystems). The observations listed by Fernando *et al*. (2003a) were followed as guidelines for scoring alleles from electropherograms.

The lab protocols (for extractions/PCRs/genotyping) listed above have been used with very high success rates in the past to examine social structure, population genetic structure, and phylogeography of Asian elephants using dung-extracted DNA (Fernando and Lande 2000, Fernando *et al*. 2000, Fernando *et al*. 2003b, Vidya and Sukumar 2005a, Vidya *et al*. 2005a,b, 2007, 2009).

Data analysis

After all the genotypes were scored, we had a dataset of multiple genotypes (from the PCR repeats) per locus per sample. Genotypes at each locus were classified depending on their quality as 1) having amplified consistently multiple times, 2) having a heterozygous genotype but not both alleles amplified multiple times, 3) having one allele consistently amplified but the other allele varying across PCRs, 4) having two different homozygous genotypes consistently, 5) having only one successful amplification of any genotype, and 6) not having amplified at all. Of

the six categories above, the first three were categorized as genotypes of good quality (since there were confirmed genotypes/alleles) and only samples that had genotypes from at least five of the nine loci were included. Using the Excel Microsatellite Toolkit (Park 2001), we checked for duplications in genotypes between samples in the good quality dataset, with the criterion that up to two alleles do not match. This criterion was used since it is not uncommon, with dung-extracted DNA, to have allelic dropout during PCRs. Once the set of matching pairs was obtained from the software, each of the pairs was manually examined to find out if the pairs could have actually come from the same individual because of allelic dropout or if they were different heterozygotes confirmed by repeated PCRs. The not-so-good data were then included to see if the samples that matched based on only loci with good quality genotypes remained identical or became different when the loci with not-so-good genotypes were also included. Since the not-so-good genotypes may not be accurate data, allowances were made for mismatches in these, and a further two (or, occasionally, three depending on the type of error) allele difference at these data were tolerated as being consistent with the same genotype (see Appendix 1 for a more detailed explanation of the above). Therefore, if the assignment of matches between pairs of individuals is biased, it is biased towards a greater number of matches and hence a smaller number of unique individuals. In previous studies using dung extracted DNA, differences in one or two loci were used as the cutoff to identify matching individuals in an African elephant population (Eggert *et al*. 2007), and samples with four or fewer alleles different were reexamined and those with up to two different alleles and the same sex were considered the same individual in an Asian elephant population (Ahlering *et al*. 2011).

The dataset of unique genotypes was then used to examine if the loci were in Hardy-Weinberg equilibrium and if pairs of loci were in linkage equilibrium. These tests were done using Genepop v.3.1 (Raymond and Rousset 1995), with Markov chain parameters of 1000 dememorization steps, 100 batches, and 1000 iterations per batch. Type I errors were corrected for by applying the Bonferroni test (see Rice 1989). The probability of identity (P_{ID}) for each locus was calculated using Identity-4 (Wagner and Sefc 1999).

Capture-Mark-Recapture analysis estimation of population size

Capture-Mark-Recapture statistical approaches to genetic data

A variety of modeling frameworks exist for obtaining population size from noninvasive genetic sampling within a capture-mark-recapture (CMR) framework (Lukacs & Burnham 2005-a; Luikart *et al.* 2010). The most widely used approach in wildlife studies are closed population models implemented in software CAPTURE or MARK. These models can be robust to heterogeneity in individual capture probabilities and can also account for temporal or behavioral variation in capture and recapture probabilities. Because individual heterogeneity in capture probability is biologically likely, many studies, including Hedges *et al.* (2007) and Pollard *et al.* (2008), follow Karanth and Nichols (1998) in using the jack-knife heterogeneity estimator (Burnham and Overton 1978) to estimate abundance. However, despite the ubiquity of this approach, the Jack-knife heterogeneity model appears less robust than alternatives when data are sparse or capture probabilities low and strongly heterogeneous (Boulanger *et al.* 2002, Harmsen *et al.* 2010, Gray & Prum 2011). Finite mixture models, which approximate heterogeneity in capture probability based on unobservable group differences (Pledger heterogeneity models; Pledger 2000), may be more robust when dealing with rare species. Such models may be particularly useful for DNA-based studies because there are frequently differences in capture probability between unobservable groups, such as young and adult animals (Lukacs & Burnham 2005-a).

Capture-mark-recapture models have also been developed to specifically address issues faced with DNA-based data. Conventional mark–recapture models assume that individuals are uniquely identifiable and that there is no error in their identification. When establishing individual identity through genotypes, however, there is always the chance of an identification error. When genotyping error exists it has been shown that population size derived from models assuming no error are biased and lead to overestimation of abundance (Waits and Leberg 2000, Mills *et al*. 2000, Creel *et al.* 2003). Consequently Lukacs & Burnham (2005-b) developed a model which includes a parameter to estimate genotyping error rate and can be incorporated into standard CMR models in MARK (White 2008). This method uses the number of genotypes observed once, relative to genotypes seen more than once, to estimate genotyping error. These

models incorporate an additional parameter α – defined as the probability that an animal was correctly identified. The misidentification models of Lukacs & Burnham (2005-b) can be compared directly to standard CMR models in an information-theoretical framework i.e. by comparing Akaike Information Criteria scores (Burnham & Anderson 2002). However during genetic analysis we classified individuals with several allele-mismatches as the same individual (see Genetic data analysis) therefore significantly reducing the probabilities of individual misidentification.

Another issue with the use of standard closed CMR models to estimate abundance is that they assume demographic and geographic closure within the survey period. Closure violation is likely to result in a positive bias in population estimates as animals moving in and out of the study area inflate the number of marked animals and negatively bias capture-probability estimates. In the majority of CMR studies on large, long-lived mammals, such as elephants and large carnivores, the sampling period is generally sufficiently short that the assumption of demographic closure (i.e. no births or deaths within the sample population) is reasonable. However violation of the assumption of geographic closure (i.e. no animals move in or out of the study area during sampling) is much more likely.

A number of statistical tests exist for examining population closure within capture-mark-recapture datasets. However the two most widely used tests are potentially biased by capture-probability variation i.e. individual heterogeneity, time or behavior effects. The widely used (e.g. by Pollard *et al.* 2008) test developed by Otis *et al.* (1978), and implementable in software CAPTURE, whilst robust to individual heterogeneity in capture probabilities may falsely reject the hypothesis of an open population in the face of temporal or behavioral variation in capture probabilities. Stanley & Burnham (1999) developed an additional closure test which compares the null hypothesis of a closed population with time dependent capture and recapture probabilities (Mt model) against the full open Jolly-Seber model. However this test, whilst appearing sensitive to permanent emigration and immigration, is less sensitive to temporary movement in and out of the study site and also performs less well when the sample size of captured individuals is less than 100 (Stanley & Burnham 1999).

A more elegant approach to testing for population closure was developed by Boulanger & McLellan (2001) and uses open Pradel models in MARK to estimate recruitment (*f*), and apparent survival rates (ψ). Assuming a population is demographically closed these parameters, recruitment and survival, correspond to immigration and fidelity. Models in which these parameters are constrained to zero and one respectively (representing a closed population) can be compared with non-constrained models (an open population) in an information-theoretical framework in MARK. This approach, though apparently robust, does not appear widely used and, in Asia, we can find only two examples of using Pradel models to test for population closure (Goswami *et al.* 2007, Harihar *et al.* 2009).

Continuous-occasion CMR models are an alternative to conventional CMR approaches for estimating abundance from fecal-DNA data (Miller *et al.* 2005, Lukacs *et al*. 2007). Such models maximize data from non-invasive sampling by considering the entire sampling period a single continuous capture–mark–recapture occasion. This allows the use of data from individuals captured multiple times within a session. Miller *et al*. (2005) developed a maximum likelihood estimator, CAPWIRE, which allows sampling with replacement to estimate abundance based on a simple urn model containing individuals of two capture probabilities. Simulation (Miller *et al.* 2005) and field studies (Robinson *et al.* 2009) have demonstrated strong performance of CAPWIRE models in comparison with conventional approaches.

Capture-Mark-Recapture methodologies

Based on observed genotypes from elephant dung samples (see Genetic analysis and laboratory methodologies) we developed capture histories for all wild elephants sampled in PPWS in a standard 'X-matrix' format with rows representing the capture histories of each captured individual (n=78) and columns representing captures on each sampling session (n=5). There were no between session recaptures of the same individual elephants from Mondulkiri Protected Forest so data from this protected area could not be analysed within a CMR framework.

We tested for closure of the PPWS population during the sampling period using the closure test of Otis *et al* (1978) in CAPTURE, the Stanley & Burnham (1999) test in software CloseTest and open Pradel models implemented in MARK (following Boulanger & McLellan 2001 and Harihar

et al. 2009). In the latter, we compared Akaike Information Criteria corrected for small sample size (AICc) scores between a model in which recruitment and survival were constrained to zero and to one, respectively (representing population closure), and an open model in which these parameters were estimated based on observed data.

The results of the closure tests detailed above suggested that abundance estimation within a closed capture mark recapture framework was reasonable (see Results below). We therefore estimated the PPWS wild elephant population size using Full Closed Captures with Heterogeneity models in MARK implementing Pledger mixture models with two mixtures of capture and recapture probabilities (White 2008). The following biologically plausible models were run:

- M0 (the null model with all capture and recapture probabilities equal);
- Mt (the time model in which capture and recapture probabilities were identical but differed between sampling sessions);
- Mh (the heterogeneity model with two mixtures of identical capture and recapture probabilities);
- Mth (the time-heterogeneity model with two mixtures of identical capture and recapture probabilities differing between sampling sessions).

Model Mb, the behaviour model in which capture and recapture probabilities differ, was not modeled as it was biologically implausible given the non-invasive fecal sampling employed in the study. Models were ranked based on AICc scores with model averaging used to produce final abundance estimates.

Although the conservative treatment of allelic mismatches meant that the probability of individual misidentification was likely to be very low we also fitted the Lukacs & Burnham (2005-b) misidentification models to the data in MARK. We tested the suitability of these models by comparing AICc scores with models in which the parameter α, the probability than an animal was correctly identified, was constrained to equal one. These constrained models are identical to the standard closed capture models (i.e. M0, Mh, Mt, Mth) described above. For each of the four models the standard CMR model, in which α was constrained to equal 1.0, was better

supported, based on AICc scores, than the misidentification models (Appendix 3). This supports the the use of a standard CMR approach in estimating elephant abundance from our data.

In order to provide comparable estimates with those from Hedges *et al.* (2007) and Pollard *et al.* (2008) we also obtained elephants abundance estimates in program CAPTURE using both the best-fitting model, based upon model selection algorithms, and the Jack-knife heterogeneity models which were used in both the previous studies. We also used CAPWIRE (Miller *et al.* 2005) to estimate the Asian elephant population in PPWS based on the total number of captures for each individual elephant. Following Miller *et al*. (2005) we implemented two models: the even capture model (ECM), which assumes equal probabilities of capture between all individuals, and the two innate rates model (TIRM) which models two mixtures of capture probabilities (analogous to Pledger heterogeneity models). A likelihood-ratio test in CAPWIRE was used to choose the most appropriate model.

Age structure and population movements

All unique individuals, as identified by the genetic analysis, were aged based on bolus circumferences using the criteria of Tyson *et al*. (2002). For individuals sampled on more than one occasion the average bolus circumference from all sampling occasions was used. Individuals were broken down into three broad age groups based on the average bolus circumferences: ≤ 30 cm = neonate + juvenile, $30 <$ circumference ≤ 42 cm = sub-adult, > 42 cm = adult (Tyson *et al.* 2002; Pollard *et al.* 2008). The geographic locations of collected samples were entered into ARCGIS and Hawth's movement extension used to analyse movements of individual elephants within, and between, PPWS and MPF.

Results

Field Surveys

During the five sampling sessions a total of 270 elephant fecal-DNA samples were collected by the four field teams (Table 2); 226 samples were collected from PPWS and 44 from MPF. Between 34 and 92 (mean 54) samples were collected per sampling session with between 34 and 119 samples (mean 68) collected per survey team (Table 2). A total of 199 samples (74%) were classified, using MIKE criteria, as fresh with 71 (26%) reasonably fresh.

Survey Team	**Ses 1**	**Ses 2**	**Ses 3**	**Ses 4**	**Ses 5**	**Total**
Kohnhek	0	0	34	0	0	34
Die Ey	19	16	0	48	36	119
Memang	20	25	0	8	20	73
MPF	8	0	0	36	0	44
Total	***47***	***41***	***34***	***92***	***56***	***270***

Table 2. Number of elephant fecal-DNA samples collected per sampling session, and by each of four sampling teams, in PPWS and MPF.

In Phnom Prich Wildlife Sanctuary all elephant fecal-DNA samples were obtained from approximately 170-km^2 centered on the Kranchilok semi-evergreen forest patch and including six of the designated hot-spots (Fig. 3). No samples were collected from other hot-spots or survey blocks despite surveying these areas. The majority of the samples from PPWS (201 samples, or 89%) were collected from four areas namely:

A. Phnom Kongshall (34 samples; 15%; only in sampling session 3)

B. North-west Krangchilok and areas north to Phnom Namkong (88 samples; 39%)

C. East Krangchilok along the lower O Katoh (26 samples; 12%)

D. South of Krangchilok along the upper O Katoh (53 samples; 23%).

The additional samples (25; 11%) were collected from other locations to the west and north of Krangchilok by field teams following-up recent reports of Asian elephant activity (Fig. 3).

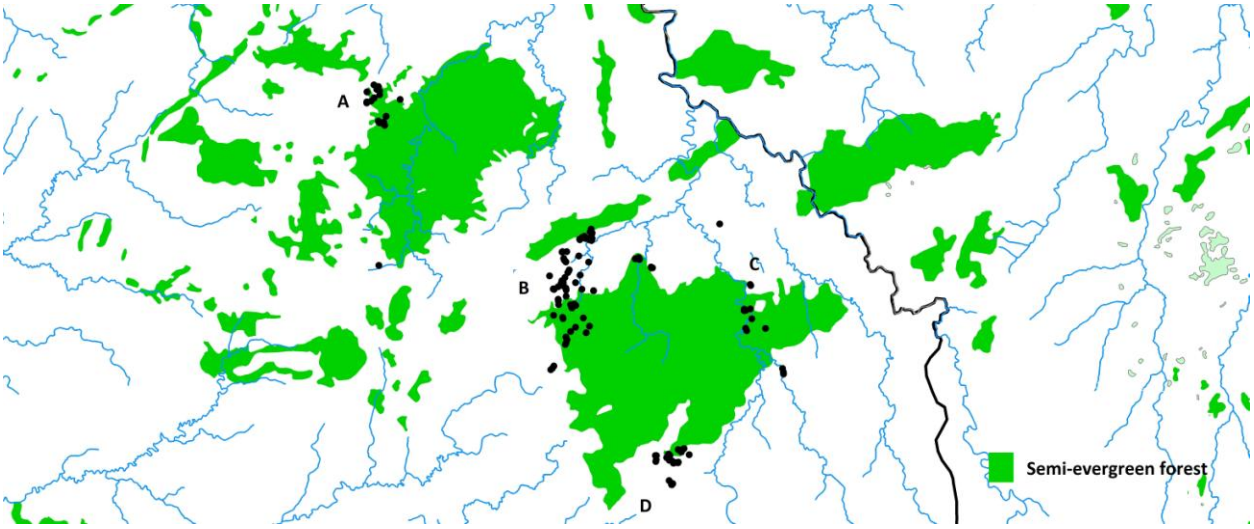

Figure 3. Locations of Asian elephant-fecal DNA samples (black-dots) collected from PPWS.

In Mondulkiri Protected Forest all samples were collected from around the Phnom Yangke and Phnom Yangpo hot-spots and an area to the west (total area approximately 50-km^2) with no

samples collected from the Trapeang Bramat or Phnom Lungchong hot-spots or the survey blocks (Fig. 4).

In addition to the 270 samples collected during field surveys 28 samples were obtained from domestic elephants known to access the two protected areas. Sixteen samples were collected from elephants in villagers in and around MPF (Dei Ey, Bousra, Pourepeth, Sre Huy) including elephants used by enforcement teams within the protected area with 12 samples from villages around PPWS (Memang and Laoka village clusters).

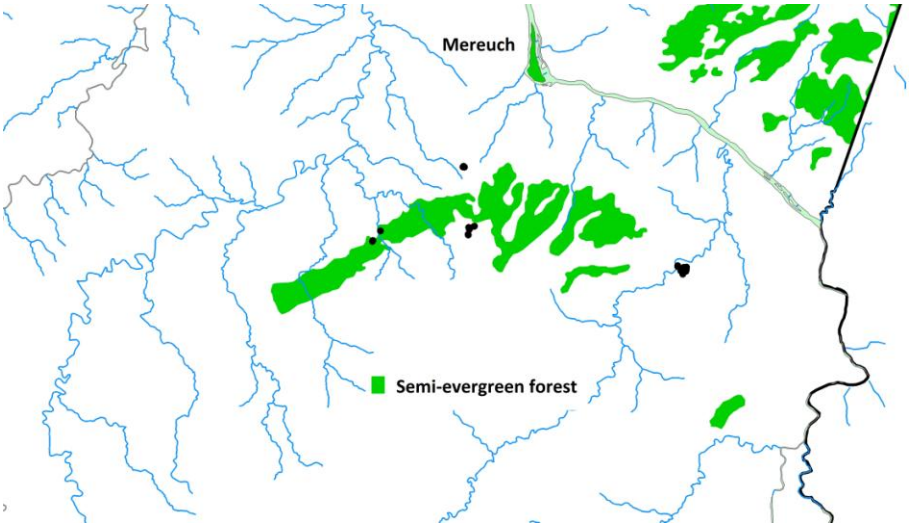

Figure 4. Locations of Asian elephant-fecal DNA samples (black-dots) collected from PPWS.

Genetic results

Average amplification success across all loci and samples was 68% with successful amplification from 194 samples corresponding to 112 unique genotypes. However there was a large variation across sampling sessions (Table 3) with sampling sessions 4 and 5 showing the lowest amplification rates across all loci. Amplfication rates also varied considerably between the four collection teams varying from 97% (Kohnhek team n=34 samples) to 50% (MPF team n= 44 samples).

	Sample size	Emu12	Emu04	FH94	Emu10	Emu14	Emu07	Emu15	Emu17	Emu03	Average
All	298	69.1	69.5	59.4	78.0	78.4	62.8	70.6	60.1	66.6	68.3
I sampling	46	79.3	83.7	69.6	88.0	91.3	67.4	81.5	71.7	79.3	79.1
II sampling	41	84.1	87.8	82.9	92.7	90.2	76.8	79.3	72.0	85.4	83.5
III sampling	34	91.2	91.2	92.6	95.6	97.1	83.8	97.1	92.6	86.8	92.0
IV sampling	92	54.3	53.8	40.2	58.7	61.4	50.5	54.3	42.4	48.4	51.6
V sampling	57	60.5	56.1	44.7	75.4	67.5	58.8	62.3	49.1	56.1	59.0
VI sampling - captive ele	28	69.6	73.2	62.5	89.3	96.4	58.9	80.4	66.1	76.8	74.8

Table 3. Percentage amplification success at the different loci for all samples and for samples collected during different sampling sessions.

The number of alleles per locus varied from 5-7, and the observed heterozygosities were also not very different across loci, with the exception of EMU17, which had an unusually high observed heterozygosity of 0.89 (Table 4).

Locus	n	No. alleles	Allele range (bp)	P_{ID}	Obs H	HWE P	Null allele freq.
EMU03	192	5	132-140	0.206	0.531	0.012	0.048
EMU04	198	5	97-105	0.350	0.384	0.129	0.039
EMU07	176	7	106-124	0.162	0.602	0.479	0.018
EMU10	204	6	94-106	0.226	0.392	0.000*	0.093
EMU12	200	5	139-158	0.328	0.580	0.570	-0.040
EMU13	82	6	100-110	-	-	-	-
EMU14	202	7	127-141	0.127	0.545	0.004*	0.084
EMU15	192	6	142-154	0.157	0.583	0.372	0.026
EMU17	164	7	118-134	0.055	0.890	0.026	-0.042
FH94	174	7	215-229	0.055	0.563	0.000*	0.119

Table 4. Sample size of alleles (n), the number of alleles, allelic range, probability of identity (P_{ID}), observed heterozygosity, P value for Hardy-Weinberg equilibrium test, and the estimated null allele frequency for the microsatellite loci used. Asterisks mark loci that are not in Hardy-Weinberg equilibrium after Bonferroni correction (Bonferroni corrected P value = 0.0056).

Three loci were not found to be in Hardy-Weinberg equilibrium, EMU10 and FH94, which were highly significant, and EMU14, which was borderline significant after Bonferroni corrections. EMU14 and FH94 did not show any departure from Hardy-Weinberg equilibrium in a southern Indian elephant population (Nandini Shetty and TNC Vidya, unpublished data) or in a population in Lao (Ahlering *et al*. 2011), but EMU10 showed departure from Hardy-Weinberg equilibrium in the Lao population (Ahlering *et al*. 2011). FH94 and EMU10 also had the highest expected null allele frequencies. It is possible that there are null alleles at FH94 and EMU10 in the Cambodian population, in which case, the number of unique genotypes will be higher than what we find at present. Only one (EMU04 and FH94) out of 36 pairs of loci showed linkage disequilibrium ($P<0.001$). Allelic ranges and P_{ID} values are shown in Table 4. The total exclusion probability was 0.995. The total P_{ID} across loci was 5.32×10^{-8} and the total P_{ID}(sib) was 1.91×10^{-3}, which are sufficiently low to prevent different individuals from being wrongly identified as the same individual. Allele frequencies at different loci are shown in Appendix 2.

Capture Histories

The 112 unique individual genotypes identified from samples by genetic analysis corresponded to 14 domestic elephants (three of which were also sampled during field surveys) and 98 wild elephants. Twenty-one individual wild elephants were recorded from Mondulkiri Protected Forest and 78 from Phnom Prich Wildlife Sanctuary. These figures include one individual caught in both PPWS (during sessions 1, 2 and 3) and MPF (during session 4). In MPF there were no between session recaptures, though one individual was sampled twice during session four. Therefore conventional CMR analysis was not possible and the number of sampled individuals (21) must be used for a minimum population estimate for this site. In PPWS 47 individual elephants were caught once, 14 twice, six three times and single individuals on five, six, seven and eight occasions. Excluding within-session re-captures a total of 55 individual elephants were caught during a single sampling session, 18 individuals during two sessions and 5 individuals during 3 sessions (Table 5).

	Ses 1	**Ses 2**	**Ses 3**	**Ses 4**	**Ses 5**
# of samples collected	39	41	34	55	55
# of Samples genotyped (%)	28 (72)	34 (83)	33 (97)	21 (38)	26 (47)
# of unique individuals	25	26	16	16	19
# of unique individuals captured for the first time	25	20	8	10	12
Number of in session re-captures	8	7	17	4	6
Number of between session re-captures	0	6	8	6	7

Table 5. Number of elephant fecal-DNA samples collected and number of unique individual elephants captured and re-captured during each CMR sampling session in PPWS.

Testing for population closure

Statistical tests for population closure in PPWS during the sampling period were inconclusive. Whilst the Stanley & Burnham (1999) test suggested population closure (χ^2 = 7.1; df-6; p = 0.31) the Otis *et al* (1978) test, in CAPTURE, rejected the hypothesis of a closed population (z = 1.7; p = 0.04). In MARK the open Pradel model estimated survivorship (θ) at 0.79 ± SE 0.1 and recruitment (*f*) at 0.15 ± SE 0.03. However the constrained Pradel model, in which θ was set at 1.0 and *f* at 0.0 (the closed model), was better supported than the open model (Δ AICc 0.84). Therefore there appears to be enough evidence of population closure to justify analysis within a closed CMR framework. However effects of possible closure violation on final population estimates need to be considered and it is important to view population estimates as likely referring to an overall 'super-population' using the landscape (Boulanger & McLellan 2001) rather than an Asian elephant population restricted to the PPWS core.

CMR abundance estimates for PPWS Asian elephant populations

Using Full Closed Captures with Heterogeneity models in program MARK (henceforth standard CMR models) M0 was the best supported model, based on AICc scores, with the PPWS Asian elephant population estimated at 136 ± SE 18 individuals (Table 6). Additional models were ranked Mh>Mt>Mht with mean population estimates of between 135 and 145 individuals (Table 5). The model averaged ***Asian elephant population estimate for PPWS was 136 ± SE 18 individuals with a 95% confidence interval range of 100 to 172***.

Model	AICc	Akaike Weight (Wi)	N ± SE
M0	-117.5	0.66	136 ± 18
Mh	-115.4	0.23	136 ± 18
Mt	-114.0	0.11	135 ± 18
Mht	-108.7	<0.01	143 ± 29

Table 6. Asian elephant population estimates (N ± standard error) for PPWS based on Full Closed Captures with Heterogeneity models in MARK. Table indicates the small-sample size Akaike Information Criteria score (AICc) and Akaike Weight for each model.

In program CAPTURE model M0 was also best-supported (rank 1.0) followed by model Mh (rank 0.81). The former model gave a very similar population estimate, 136 ± SE 18, to the equivalent model in MARK but with slightly different 95% confidence intervals (110-183). However using the Mh Jack-knife estimator, the default estimator for CMR studies in Asia (e.g. Hedges *et al.* 2007, Pollard *et al*. 2008), gave an estimate of 165 individuals ± SE 18 with approximate 95% confidence intervals of 136 to 208. In contrast to models in CAPTURE and MARK the model selection tests in program CAPWIRE supported the use of the two innate rates model (TIRM) over the even capture model (ECM), which assumes equal probabilities of capture for all individuals(Appendix 4). Using the TIRM model the Asian elephant population estimate for PPWS was between ***105 and 175 individuals (95% confidence interval range) with a mean estimate of 154***.

Age structure of the Asian elephant population

Based on mean bolus circumferences of all unique individuals the Asian elephant population in both PPWS and MPF consists of a high proportion of sub-adults (45%) and adults (43%) (Table 7). No neonate or juvenile samples were collected from MPF where the proportion of adults to sub-adults was also higher than in PPWS.

Bolus Circumference	Age Class	Number (and %) of individuals PPWS	Number (and %) of individuals MPF
≤ 30 cm	Neonate & Juvenile	11 (14)	0
30 – 42 cm	Sub-adult	36 (46)	9 (43)
≥ 42 cm	Adult	31 (40)	12 (57)

Table 7: Age structure of the wild Asian elephant population in PPWS and MPF based on mean bolus circumferences (using criteria of Tyson et al. 2002)

Asian elephant movement patterns within the Eastern Plains Landscape

A total of 23 Asian elephants (29% of all captured individuals in PPWS) were recaptured, either between or within sessions, more than 2-km away from their original capture locations in PPWS. There was clear evidence of movements of individual elephants between the four 'clusters' of locations from which samples were collected (Fig. 5). Four individual elephants were recorded moving between three of the 'clusters' with an additional 15 moving between two 'clusters' and four individuals being recaptured more than 2-km away from their original locations but remaining within the north-west Krangchilok/Phnom Namkong area. In addition one individual, aged as an adult based on bolus circumferences, moved between PPWS and MPF (Fig. 6). This individual was capture twice during session one from the east of the north-west Kranchilok/Phnom Namkong area before being recaptured twice, approximately 7-km to the

south-east, around lower O'Katoh in session two and on two additional occasions a further 17-km to the north-west in Phnom Konshall during session three. Finally during session four, 23-days latter, the same individual was captured 59-km north-east in MPF along O'Lumith.

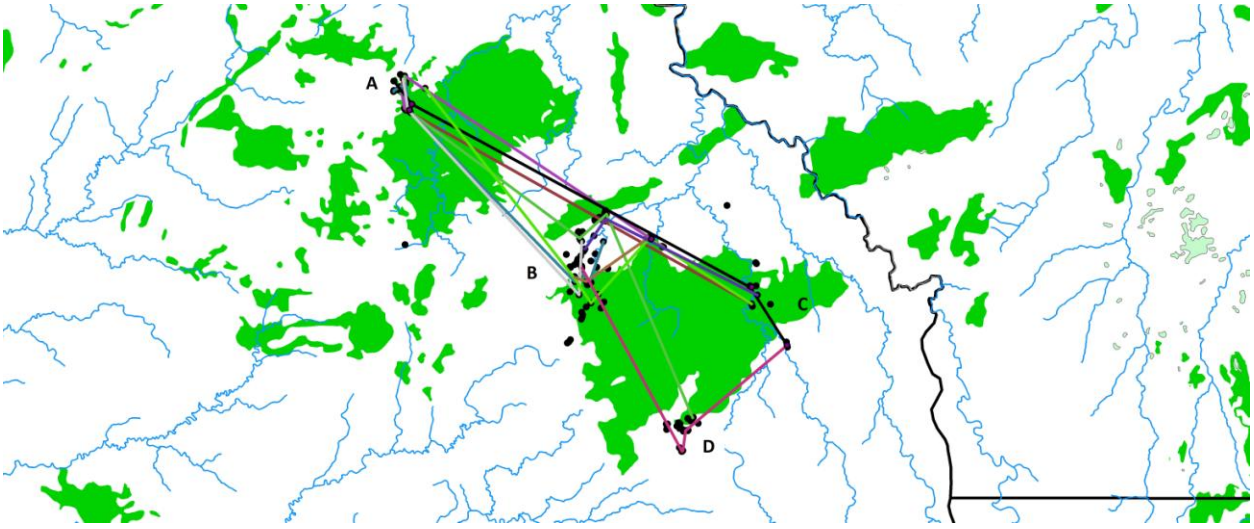

Figure 5. Individual elephant movements (linked by different colored lines) in and around Kongchilok evergreen forest block PPWS. Letters refer to 'clusters' of elephant samples.

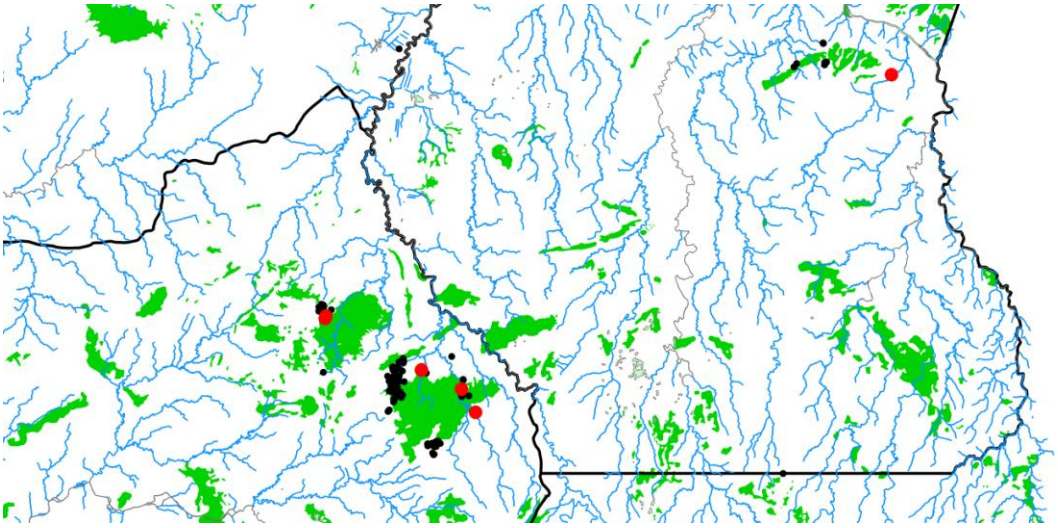

Figure 6. Capture and re-capture locations (red-dots) of an adult Asian elephant which moved between PPWS and MPF. Black-dots locations of all samples collected.

Discussion

Robust monitoring is essential to effectively document the success or failure of conservation activities (Sutherland *et al.* 2003, Nichols & Williams 2006, Pullin & Knight 2009). However in tropical Asia few biological monitoring programs exist (but see but see O'Kelly & Nut 2010; Ryan *et al*. 2011, Gray *et al.* 2011) and robust population baselines are only available for a few focal species in selected priority protected areas (e.g. Jathana *et al.* 2003, Goswami *et al.* 2007, Harihar *et al.* 2009). Despite Asian elephant being a flagship species for conservation across tropical Asia there are few reliable population estimates from anywhere within the species' global range (Blake & Hedges 2004). In this study we provide the first robust baseline population estimates for Asian elephant from Phnom Prich Wildlife Sanctuary and Mondulkiri Protected Forest, two protected areas in Cambodia's Eastern Plains Landscape. This baseline forms part of the WWF Greater Mekong 2011-2015 Strategic Plan monitoring system and plans are for surveys to be repeated in 2013/14, 2015/16 and 2019/20.

The results from this study, and that of Pollard *et al.* (2008), suggest that the Eastern Plains Landscape may support the largest wild Asian elephant population in Cambodia and possibly Indochina (*sensu* Laos, Cambodia, Vietnam). There may, however, be some duplication of individuals between Seima Protected Forest and PPWS due to movements of elephants between the protected areas. During their 2006 study Pollard *et al.* (2008) obtained a number of samples from north-east Seima Protected Forest adjacent to PPWS during their 4th sampling period, in late April, but not during early sampling sessions. They concluded that these elephants may have dispersed into Seima Protected Forest from PPWS. In addition the PPWS estimate is best regarded as referring to the 'super-population' which uses the protected area during the dry-season due to evidence of marginal closure violations. Despite this the PPWS estimate of 100-172 individuals, together with that from the adjacent Seima Protected Forest of 101-139 individuals (Pollard *et al.* 2008), suggests that the landscape is critical for Asian elephant conservation. Our findings, therefore, provide further support for the global significance of the Eastern Plains Landscape, and PPWS and MPF specifically, for large mammal conservation

(Phan & Gray 2010, O'Kelly & Nut 2010, Gray & Phan 2011, Gray & Prum 2011, Gray *et al.* 2011).

All elephant fecal-DNA samples from PPWS were collected from a restricted area, of approximately 170-km^2, in, and around, the Krangchilok semi-evergreen forest block and Phnom Namkong and Phnom Konsall to the north. This supports the previous suggestions (A Maxwell pers. obs., WWF-internal data, including ranger and hunter reports) that this area supports the majority of the PPWS Asian elephant population during the dry-season. This area must, therefore, be regarded as a priority conservation zone in PPWS and be managed accordingly. The extent to which Asian elephant move away from this area during the wet-season is unclear. Further studies involving GPS or satellite collaring are recommended. We also document the first evidence of dispersal, by any species, between the Ministry of the Environment managed PPWS and the Forestry Administration managed MPF. This clearly illustrates that these two protected areas cannot be viewed in isolation and that landscape scale management across protected area boundaries is essential. Maintaining connectivity between semi-evergreen forest patches in south-east PPWS, southern Seima Protected Forest, and eastern MPF into Yok Don National Park, Vietnam is critical for the persistence of a viable Asian elephant population in the landscape.

Based on the average bolus circumferences of unique individuals the Asian elephant population in both PPWS and MPF is biased towards sub-adults (45%) and adults (43%). No juvenile/neonates were recorded from MPF with 11 (14%) juvenile/neonates in PPWS. However these results must be treated with some caution as the defecations rates of juveniles are usually lower than those of sub-adult and adult elephants (TNC Vidya pers. obs.). In addition there appears to be considerable inter-individual variation in bolus circumference of known aged domestic Asian elephants in Mondulkiri (J. Haywood pers. comm.). The age structure from PPWS/MPF is very similar to that reported by Pollard *et al.* (2008) from the adjacent Seima Protected Forest (11% juvenile/neonates; 38% sub-adult; 51% adult) and by Hedges *et al.* (2007) from the Nakai plateau, Laos (20% juvenile/neonates; 36% sub-adult; 44% adult).

The different statistical CMR approaches to estimating the PPWS Asian elephant population gave generally similar results. However the mean estimate from the Jack-knife heterogeneity model in CAPTURE, the approach used in both previous Asian elephant CMR fecal-DNA studies (Hedges *et al.* 2007, Pollard *et al*. 2008), was >20% higher than that from model-averaging in MARK. Although true population size is unknown, and therefore bias of estimates cannot be inferred, the unthinking use of the Jack-knife heterogeneity estimator for calculating abundance has been criticised (Boulanger *et al.* 2002, Harmsen *et al.* 2010, Gray & Prum 2011). We therefore recommend the use of information theoretic methods to formally compare a subset of biologically plausible models in MARK when estimating abundance in fecal-DNA CMR studies.

The PPWS elephant population showed evidence of marginal closure violation during the study period. Thus abundance estimates from closed population CMR models must be viewed with some caution. However when the assumption of geographic closure is violated it is difficult to robustly estimate abundance, the key parameter of interest in conservation monitoring of rare and globally threatened species, using current CMR techniques. POPAN models (Schwarz & Arnason 1996) have been used for estimating 'super-population' size in Asian elephant in India (Goswami *et al.* 2007) and in a variety of marine animals (e.g. Rowat *et al.* 2009) when populations are not closed. However this approach is constrained in that it only allows animals to enter or leave a population once (i.e. deaths/births and permanent emigration or immigration). Therefore whilst a useful approach for estimating abundance in long-term studies where violations of the assumption of demographic closure are likely POPAN models are likely to be less robust when populations are geographically open i.e. animals moving in and out of the study area during the survey period. Recent advances in CMR statistics, particularly robust mark-resight models which are also able to account for within-session recaptures, appear a promising approach for estimating abundance in open populations though more research, and ideally a large data-set, is needed (McClintock 2009, Ryan *et al.* 2011).

The overall successful amplification rate of our samples (68%) was lower than some previous similar work on the Asian elephant: 99% amplification in Fernando *et al.'s* (2003a) study and 86-97% in Vidya *et al.'s* (2005a) study, both of which used exactly the same lab protocols as those in the present work, and 81% amplification from Pollard *et al.*'s (2008) and Hedges *et al.*'s (2007) studies. However, our amplification rates varied greatly between sampling sessions and sampling teams (see Results).The overall low amplification rates appeared to be a result of poor sample quality as the electropherograms had lower intensities than those of the positive controls which were also dung-extracted DNA samples, but collected within a few hours of observed defecation from elephants in southern India. The low quality of samples could be due to several reasons:

1. The samples collected may not have been really fresh at the time of collection. It would be desirable to classify samples as "fresh" only if they are about a few hours old, but MIKE guidelines allow samples less than two days old to be classified as fresh, and it is often not easy to differentiate between samples that are two days old and 3-4 days old. Dung collected within a few hours or on the same day (<24 hours) gives the best DNA extracts (Vidya and Sukumar 2005b, TNC Vidya pers. obs.). Once fresh dung has been collected into an appropriate storage medium such as ethanol, storing the dung for a year does not affect PCR amplification, and storing the dung for about five years has also given high amplification success rates (TNC Vidya pers. obs.).
2. The volume of buffer in the sample tubes may not have been sufficient to preserve the volume of dung collected. There is often a tendency to collect as much dung as possible and this leads to improper storage (even if the dung collected is very fresh). While twice the volume of dung is sufficient if ethanol is used as the storage medium, many times the volume of dung is probably required when buffer is used. Based on results from dung samples from India (see Vidya and Sukumar 2005b), we recommend 95% ethanol as the storage medium for dung samples rather than buffer. However, since ethanol is inflammable, it may not be logistically feasible to use if samples have to be flown across countries.
3. The buffer itself may not have been of good quality. By the time they arrived at the JNCASR, Bangalore many of the samples had some white precipitate at the bottom, which means that some constituent of the buffer had precipitated out, and that might have affected

storage. However, this issue with the buffer is probably minor compared to the collection itself.

4. Another, more important, problem could be that not all samples may have been collected from the outer layer of dung. There were predominantly large chunks of fibre in many of the samples collected, which would not have been present if only the outer layer of dung had been collected.

We recommend that all these factors are explored, using preliminary genetic analysis, prior to any future fecal-DNA CMR studies in the landscape in order to identify the main causes of low amplification rates. Based on the results of such preliminary studies, future sample collection and storage protocols should be modified. Low amplification rates, and thus low numbers of samples successfully genotyped, are likely to lead to reduced precision in population estimates due to decreased individual capture and re-capture probabilities (Gray & Prum 2011). A final future survey recommendation, based on this study, is the importance of collecting samples from all domestic elephants likely to use the study area. Whilst survey teams were instructed not to collect samples that obviously came from domestic animals (e.g. when dung accompanied by distinctive signs of chains of domestic elephant) three field samples came from domestic elephants. Including these samples in CMR analysis would have increased the estimated population size by approximately 8%.

Acknowledgements

This study forms part of WWF Greater Mekong Cambodia Country Program's Eastern Plains Landscape Project and was primarily funded by United Stated Fish and Wildlife Service grant # 98210-8-G567. Additional funding was provided by WWF International AREAS program, WWF-US, and Humanscale. Work in Phnom Prich Wildlife Sanctuary is with permission of the Ministry of the Environment and support from His Excellency Chay Samith, Samrangdy Vicheth and Han Sakhan. Work in Mondulkiri Protected Forest is with permission of the Forestry Administration of the Ministry of Agriculture Forestry and Fisheries with support from His Excellency Cheng Kimsun, Men Phymean, Keo Omaliss, Song Keang and Keo Sopheak. This project would not have been possible without the hard-work of the four field teams: Sary Tre and Dong Kit (Memmang), Sem Pros and Yet Sreng (Kohnhek), Ngoeum Naroeun and Gkill (Dei Ey), and Kham Mephoeung and Mel Trock (MPF). We thank JNCASR for laboratory facilities and logistics, and Nandini Shetty, Kanika Mendiratta and Anitha CA, of the JNCASR sequencing facility, who assisted with genetic laboratory analysis. TNCV was supported by the DST-Ramanujan Fellowship and JNCASR during this work. Craig Bruce, Samang Ang, Seng Teak, and Nick Cox provided logistical and administrative support whilst Christy Williams, Simon Hedges, Ed Pollard, Shannon Barber-Meyer and Barney Long assisted in study design, survey objectives and provided statistical advice.

References

Ahlering, M.A., Hedges, S., Johnson, A., Tyson, M., Schuttler, S.G. & Eggert, L.S. 2011. Genetic diversity, social structure, and conservation value of the elephants of the Nakai Plateau, Lao PDR, based on non-invasive sampling. *Conservation Genetics* 12: 413-422.

Amos, W., Whitehead, H., Ferrari, M. J., Payne, R. and Gordon, J. 1992. Restrictable DNA from sloughed cetacean skin: its potential for use in population analyses. *Marine Mammal Science* 8, 275–283.

Arandjelovic, M., Head, J., Kuhl, H., Boesch, C., Robbins, M.M., Maisels, F., Vigilant, L. 2010. Effective non-invasive genetic monitoring of multiple wild western gorilla groups. *Biological Conservation* 143: 1780-1791.

Barnes, R.F.W. 2002. The problem of precision and trend detection posed by small elephant populations in West Africa. *African Journal of Ecology* 40: 179–185.

Bellemain, E., Swenson, J.E., Tallmon, D., Brunberg, S. & Taberlet, P. 2005. Estimating population size of elusive animals with DNA from hunter-collected feces: four methods for Brown Bears. *Conservation Biology* 19: 150-161.

Blake, S. & Hedges, S. 2004. Sinking the flagship: the case of forest elephants in Asia and Africa. *Conservation Biology* 18: 1191–1202.

Boulanger, J. & McLellan, B. 2001. Closure violation in DNA-based mark–recapture estimation of grizzly bear populations. *Canadian Journal of Zoology* 79: 642-651.

Boulanger, J., G. C. White, B. N. McLellan, J. Woods, M. Proctor & Himmer S. 2002. A meta-analysis of grizzly bear DNA mark-recapture projects in British Columbia, Canada. *Ursus* 13: 137-152.

Boulanger, J., Kendall, K.C., Stetz, J.B., Roon, D.A., Waita, L.P. & Paetkau, D. 2008. Multiple data sources improved DNA-based Mark-recapture populations estimates of grizzly bears. *Ecological Applications* 18: 577-589.

Burnham, K.P. & Anderson, D.R. 2002. *Model Selection and Multi-model Inference*. Springer, New York, USA.

Burnham K.P. & Overton, W.S. 1978. Estimation of the size of a closed population when capture probabilities vary among animals. *Biometrics* 65:625–633

Choudhury, A., Lahiri Choudhury, D.K., Desai, A., Duckworth, J.W., Easa, P.S., Johnsingh, A.J.T., Fernando, P., Hedges, S., Gunawardena, M., Kurt, F., Karanth, U., Lister, A., Menon, V., Riddle, H., Rübel, A. & Wikramanayake, E. 2008. *Elephas maximus*. In: IUCN 2011. IUCN Red List of Threatened Species. Version 2011.1. <www.iucnredlist.org>.

Comstock K.E., Wasser S.K. & Ostrander E.A.. 2000. Polymorphic microsatellite DNA loci identified in the African elephant (*Loxodonta africana*). *Molecular Ecology* 9: 1004-1005.

Creel, S.G., Spong, G., Sands, J.L., Rotella, J., Zeigle, L., Murphy, J.K.M & Smith, D. 2003. Population size estimation in Yellowstone wolves with error-prone noninvasive microsatellite genotypes. *Molecular Ecology* 12:7:2003-2009.

Cubaynes, S., Pradel, R., Choquet, R., Duchamp, C., Gaillard, J.M., Lebreton, J.D., Marboutin, E., Miquel, C., Reboulet, A.M., Poillot, C., Taberlet, P. & Gimenez, O. 2010. Importance of Accounting for Detection Heterogeneity When Estimating Abundance: the Case of French Wolves. *Conservation Biology* 24: 621-626.

Desai, A.A., & V. Lic. 1996. *Status and distribution of large mammals in eastern Cambodia: Results of the first foot surveys in Mondulkiri and Ratanakiri*. IUCN/FFI/WWF. Phnom Penh, Cambodia.

Desai, A.A., Chak S., Ou R., Lic V. & A. Maxwell. 2002. *Initial Surveys to Determine the Distribution of Wild Asian Elephant Populations in Selected Areas of Northeastern Cambodia, 2000-2001*. WWF Cambodia, WWF Indochina Programme Office, Phnom Penh.

Duckworth, J.W. & S. Hedges. 1998. *Tracking Tigers: A review of the status of tiger, Asian elephant, gaur and banteng in Vietnam, Lao, Cambodia and Yunnan (China).* WWF Indochina, Hanoi, Vietnam.

Eggert, L.S., Eggert, J. A., & Woodruff, D. S. 2003. Estimating population sizes for elusive animals: the forest elephants of Kakum National Park, Ghana. *Molecular Ecology* 12: 1389–1402.

Eggert, L.S., Patterson, G. & Maldonado, J.E. 2007. The Knysna elephants: a population study conducted using faecal DNA. *African Journal of Ecology* 46: 19-23.

Fernando, P. & Lande, R. 2000. Molecular genetic and behavioural analysis of social organization in the Asian elephant (*Elephas maximus*). *Behavioural Ecology and Sociobiology* 48: 84–91.

Fernando, P., Pfrender, M., Encalada, S. & Lande, R. 2000. Mitochondrial DNA variation, phylogeography and population structure of the Asian elephant. *Heredity* 84: 362–372.

Fernando, P., Vidya, T.N.C., Rajapakse, C., Dangolla, A. & Melnick, D.J. 2003a. Reliable non-invasive genotyping: fantasy or reality? *Journal of Heredity* 94: 115-123.

Fernando, P., Vidya, T.N.C., Payne, J., Stuewe, M., Davison, G., Alfred, R.J., Andau, P., Bosi, E., Kilbourn, A. & Melnick, D.J. 2003b. DNA analysis indicates that Asian elephants are native to Borneo and are therefore a high priority for conservation. *PLoS Biology* 1: 110-115.

Goswami, V.R., Madhusudan, M.D. & Karanth, K.U. 2007. Application of photographic capture–recapture modelling to estimate demographic parameters for male Asian elephants. *Animal Conservation* 10: 391-399.

Gray, T.N.E., and Phan, C. 2011. Habitat preferences and activity patterns of the larger mammal community in Phnom Prich Wildlife Sanctuary, Cambodia. *The Raffles Bulletin of Zoology* 59: 311-318.

Gray, T.N.E. & Prum, S. 2011. Leopard density in a post-conflict landscape Cambodia: evidence from spatially explicit capture-mark-recapture. *Journal of Wildlife Management* DOI: 10.1002/jwmg.230

Gray, T.N.E., Pin, C. and Pin, C. 2010. Status and ecology of Large-spotted Civet in eastern Cambodia. *Small Carnivore Conservation* 43: 12-15.

Gray, T.N.E., Phan, C., Pin, C & Prum, S. 2011. *Establishing baseline ungulate densities in Mondulkiri Protected Forest and Phnom Prich Wildlife Sanctuary, Cambodia*. WWF Greater Mekong Cambodia Country Program, Phnom Penh.

Gray, T.N.E., Phan, C. Pin, C. & Prum, S. submitted. The ecology and conservation of jungle cat *Felis chaus* and sympatric cats in Cambodia's Eastern Plains Landscape. *Oryx*.

Gray, T.N.E., Pollard, E.P., & Evans, T.D. in prep. Birds of Mondulkri, Cambodia: distribution, status and conservation.

Harihar, A., B. Pandav & Goyal, S.P. 2009. Density of leopards (*Panthera pardus*) in the Chilla Range of Rajaji National Park, Uttarakhand, India. *Mammalia* 74: 83-87.

Harmsen, B.J., R.J. Foster & Doncaster, C.P. 2011. Heterogeneous capture-rates in low density populations and consequences for capture-recapture analysis of camera-trap data. *Population Ecology* 53:1: 253-259.

Hedges, S. & Tyson, M.J. 2002. *Some thoughts on counting elephants in SE Asian forests, with particular reference to the CITES Monitoring the Illegal Killing of Elephants Program*. Wildlife Conservation Society, Bogor, Indonesia.

Hedges, S. & Lawson, D. 2006. *Dung Survey Standards for the MIKE Programme*. CITES MIKE Programme, Central Coordinating Unit, PO Box 68200, Nairobi, Kenya.

Hedges, S., Tyson, M.J., Sitompul, A.F., Kinnaird, M.F., Gunaryadi, D., & Aslan. 2005. Distribution, status, and conservation needs of Asian elephants (*Elephas maximus*) in Lampung Province, Sumatra, Indonesia. *Biological Conservation* 24: 35–48.

Hedges, S., Johnson, A., Tyson, M., & Eggert, L. 2007. *Determination of the size and distribution of populations of elephants, assessment of habitat and resource use, and assessment and management of human–elephant conflicts on the Nakai Plateau and surrounding areas*: Wildlife Conservation Society – Lao Program, Vientiane.

Jacob, G., Debrunner, R., Gugerli, F., Schmid, B. & Bollmann, K. 2010. Field surveys of capercaillie (*Tetrao urogallus*) in the Swiss Alps underestimated local abundance of the species as revealed by genetic analyses of non-invasive samples. *Conservation Genetics* 11: 33-44.

Jathanna, D., Karanth, K.U. & Johnsingh, A.J.T. 2003. Estimation of large herbivore densities in the tropical forests of southern India using distance sampling. *Journal of the Zoological Society London* 261: 285-290.

Karanth, K.U., & J.D. Nichols.1998. Estimation of tiger densities in India using photographic capture and recaptures. *Ecology* 79:2852-2862.

Kongrit C., Siripunkaw C., Brockelman W.Y., Akkarapatumwong V., Wright T.F., & Eggert L.S. 2008. Isolation and characterization of dinucleotide microsatellite loci in the Asian elephant (*Elephas maximus*). *Molecular Ecology Resources* 8: 175-177.

Laing, S. E., Buckland, S. T., Burn, R. W., Lambie, D., & Amphlett, A. 2003. Dung and nest surveys: estimating decay rates. *Journal of Applied Ecology* 40: 1102–1111.

Leimgruber, P., Gagnon, J.B., Wemmer, C., Kelly, D.S., Songer, M.A. & Selig, E.R. 2003. Fragmentation of Asia's remaining wildlands: implications for Asian elephant conservation. *Animal Conservation* 6:4: 347-359.

Long, B., Swan, S. & Kry Masphal. 2000. *Biological surveys in Northeast Cambodia, April 2000*. FFI and the Wildlife Protection Office, Hanoi & Phnom Penh, Cambodia.

Luikart, G., Ryman, N., Tallmon, D.A., Schwartz, M.K. & Allendorf, F.W. XXXX. Estimation of census and effective population sizes: the increasing usefulness of DNA-based approaches. *Conservation Genetics* DOI 10.1007/s10592-010-0050-7

Lukacs, P.M. & Burnham, K.P. 2005-a. Review of capture–recapture methods applicable to noninvasive genetic sampling. *Molecular Ecology* 14: 3909-3919.

Lukacs, P.M. & Burnham, K.P. 2005-b. Research notes: estimating population size from DNA-based closed capture–recapture data incorporating genotyping error. *Journal of Wildlife Management* 69:1: 396-403.

Lukacs, P.M., Eggert. L.S. & Burnham, K.P. 2007. Estimating population size from multiple detections with non-invasive genetic data. *Wildlife Biology in Practice* 3: 83-92.

McClintock, B. 2009. Mark-resight models. Ch 18 in Cooch, E. & White, G. *Program MARK. A gentle introduction*. <http://www.phidot.org/software/mark/index.html>.

Miller C.R., Joyce P., & Waits, L. 2005 A new method for estimating the size of small populations from genetic mark-recapture data. *Molecular Ecology* 14: 1991-2005.

Mills, L.S., Citta, J.J., Lair, K.P., Schwartz, M.K. & Tallmon, D.A. 2000. Estimating animal abundance using noninvasive DNA sampling: promise and pitfalls. *Ecological Applications* 10: 283-294.

Mondol, S., K.U. Karanth, A.M. Gopalaswarmy, A. Andheria, & U. Ramakrishnan. 2009. Evaluation of non-invasive genetic sampling methods for estimating tiger population size. *Biological Conservation* 142:2350-2360.

Nichols, J.D., & Karanth, K.U. 2002. Statistical concepts: estimating absolute densities of tigers using capture-recapture sampling. Pages 124-137 *in* K.U. Karanth, and J.D. Nichols, editors. *A Manual for Researchers, Managers and Conservationists in Tropical Asia*. Centre for Wildlife Studies, Bangalore, India.

Nichols, J.D. & Williams, B.K. 2006. Monitoring for conservation. *Trends in Ecology & Evolution* 21: 668-673

O'Kelly, H. & Nut, M. H. 2010. *Monitoring of ungulate, primate and peafowl populations using line transect surveys in Seima Protection Forest, Cambodia 2005-2010*. Wildlife Conservation Society Cambodia. Phnom Penh.

Otis, D. L., Burnham, K. P., White, G. C. & Anderson, D. R. 1978. Statistical inference from capture data on closed animal populations. *Wildlife Monographs* 62. 135 pp.

Park S.D.E. 2001. *Trypanotolerance in West African cattle and the population genetic effects of selection*. Ph.D. thesis, University of Dublin.

Phan, C. and Gray, T.N.E. 2010-a. Status and habitat of Yellow-cheeked Crested Gibbons in Phnom Prich Wildlife Sanctuary, Mondulkiri, Cambodia. pp 79-89 in Nadler, T. et al. (eds). *Conservation of Primates in Indochina*. Frankfurt Zoological Society and Conservation International.

Phan, C & Gray, T.N.E. 2010-b. Ecology and natural history of Banteng in eastern Cambodia: evidence from camera-trapping in Mondulkiri Protected Forest and Phnom Prich Wildlife Sanctuary. *Cambodian Journal of Natural History* 2: 118-126.

Pollard, E., Clements, T., Nut Meng Hor, Sok Ko & Rawson,B. 2007. *Status and conservation of globally threatened primates in the Seima Biodiversity Conservation Area, Cambodia*. WCS, Phnom Penh.

Pollard, E., Eggert, L., Cheur, C. & Hedges, S. 2008. *The status and conservation of Asian Elephant in the Seima Biodiversity Conservation Area*, Cambodia. WCS Cambodia Program, Phnom Penh.

Pledger, S. 2000. Unified maximum likelihood estimates for closed models using mixtures. *Biometrics* 56:434-442.

Pullin, A.S. & Knight, T.M. 2009. Doing more good than harm – Building an evidence-base for conservation and environmental management. *Biological Conservation* 142: 931-934.

Rawson, B.M., Clements, T. & Meng, H. 2009. Status and conservation of yellow-cheeked crested gibbons in Seima biodiversity conservation area, Mondulkiri Province, Cambodia. In *The gibbons: new perspectives on small Ape socioecology and population biology*. Lappan, S. & Whitaker, D.M. (Eds). New York: Springer.

Raymond M. & Rousset F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248-249.

Rice W. 1989. Analysing tables of statistical tests. *Evolution* 43: 223-225.

Robinson, S.J., Waits, L.P. & Martin I.D. 2009. Estimating abundance of American black bears using DNA-based capture–mark–recapture models. *Ursus* 20: 1-11.

Rowat, D., Speed, C.W., Meekan, M.G., Mauvisa, M., Gore, A, Corey, J. & Bradshaw, J.A. 2009. Population abundance and apparent survival of the Vulnerable whale shark Rhincodon typus in the Seychelles aggregation. *Oryx* 43: 591–598

Rudnick, J.A., Katzner, T.E., Bragin, E.A., Rhodes, O.E., & Dewoody, J.A. 2005. Using naturally shed feathers for individual identification, genetic parentage analyses, and population monitoring in an endangered Eastern imperial eagle (*Aquila heliaca*) population from Kazakhstan. *Molecular Ecology* 14: 2959-2967.

Ryan, G.E., Dove, V., Trujillo, F. & Doherty, P.F. 2011. Irrawaddy dolphin demography in the Mekong River: an application of mark-resight models. *Ecosphere* 2: 58.

Schwarz, C.J. & Arnason, A.N. 1996. A general methodology for the analysis of capture–recapture experiments in open populations. *Biometrics* 52, 860–873.

Seng, K.H., Pech, B, Poole, C.M., Tordoff, A.W., Davidson, P. & Delattre, E. (2003). *Directory of Important Bird Areas in Cambodia: Key Sites for Conservation*. Department of Forestry and Wildlife, Department of Nature Conservation and Protection, BirdLife International in Indochina and the Wildlife Conservation Society: Phnom Penh, Cambodia.

Stanley, T.R. & Burnham, K.P.. 1999. A closure test for time-specific capture–recapture data. Environmental *Ecological Statistics* 6:197–20

Sukumar, R. 2003. The *Living Elephants: Evolutionary Ecology, Behaviour, and Conservation*. Oxford University Press, Oxford, UK

Sutherland, W. J., Pullin, P.M., & Knight, T.M. 2004. The need for evidence-based conservation. *Trends in Ecology & Evolution* 19: 305-308.

Timmins, R.J. & Ou, R. (2001) *The Importance of Phnom Prich Wildlife Sanctuary and Adjacent Areas for the Conservation of Tigers and Other Key Species: A Summary*. Field Survey Report, WWF Cambodia Conservation Program: Phnom Penh.

Tyson, M., Hedges, S., & Sitompul, A. F. 2002. *WCS–Indonesia Program Sumatran Elephant Project: Six-month report January–June, 2002*. Unpublished report to the Wildlife Conservation Society, Bronx, NY, USA.

Vidya, T.N.C. & Sukumar, R. 2005a. Social organization of the Asian elephant (*Elephas maximus*) in southern India inferred from microsatellite DNA. *Journal of Ethology* 23: 205-210.

Vidya, T.N.C. & Sukumar, R. 2005b. Amplification success and feasibility of using microsatellite loci amplified from dung to population genetic studies of the Asian elephant (*Elephas maximus*): a field study from southern India. *Current Science* 88: 489-491.

Vidya, T.N.C., Fernando, P., Melnick, D.J. & Sukumar, R. 2005a. Population differentiation within and among Asian elephant (*Elephas maximus*) populations in southern India. *Heredity* 94: 71-80.

Vidya, T.N.C., Fernando, P., Melnick, D.J. & Sukumar, R. 2005b. Molecular genetic structure and conservation of Asian elephant (*Elephas maximus*) populations across India. *Animal Conservation* 8: 377-388.

Vidya, T.N.C., Varma, S., Dang, N.X., Van Thanh, T. & Sukumar, R. 2007. Minimum population size, genetic diversity, and social structure of the Asian elephant in Cat Tien National Park and its adjoining areas, Vietnam, based on molecular genetic analyses. *Conservation Genetics* 8: 1471-1478.

Vidya, T.N.C., Sukumar, R. & Melnick, D.J. 2009. A range-wide phylogeography of the Asian elephant (*Elephas maximus*) based on mitochondrial DNA. *Proceedings of the Royal Society of London, Series B* 276: 893-902.

Wagner, H.W. & Sefc K.M. 1999 IDENTITY 1.0. Centre for Applied Genetics, University of Agricultural Sciences, Vienna.

Waits, J.L. & Leberg, P.L. 2000. Biases associated with population estimation using molecular tagging. *Animal Conservation* 3:3: 191-199.

Webber, C.E., Tuy, S., Maltby, M.P. & Lee, P.C. 2011. Elephant crop-raiding and human–elephant conflict in Cambodia: crop selection and seasonal timings of raids. *Oryx* 45: 243-251.

White, G.C. 2008. Closed population estimation models and their extensions in Program MARK. *Environmental and Ecological Statistics* 15: 89-99.

Williams, B.K., Nichols, J.D. & Conroy, M.J. 2002. *Analysis and Management of Animal Populations*, 1st edition. Academic Press.

Wright, H.L., Collar, N.J., Lake, I.R., Net Norin, Rours Vann, Sok Ko, Sum Phearun & Dolman, P.M. (in press). First census of white-shouldered ibis *Pseudibis davisoni* reveals roost-site mismatch with Cambodia's protected areas. *Oryx*.

Appendix 1: Sample electropherograms of different loci and details of genotype assignment to samples

As mentioned in the Methods, genotypes were scored using GeneMapper software. Each genotype was manually checked after automatic scoring by the software since automatic scoring can be error-prone. Screenshot images of electropherograms of the different loci are shown on the following pages (Figures 2-14). On the Y-axes of these graphs are intensities, thus showing variations in quality of amplification. We classified peaks of intensities less than about 1500 as small peaks (low intensity) and those less than about 200 as very small (very low intensity), and such samples were reamplified. Many samples did not amplify at all and they are not shown here.

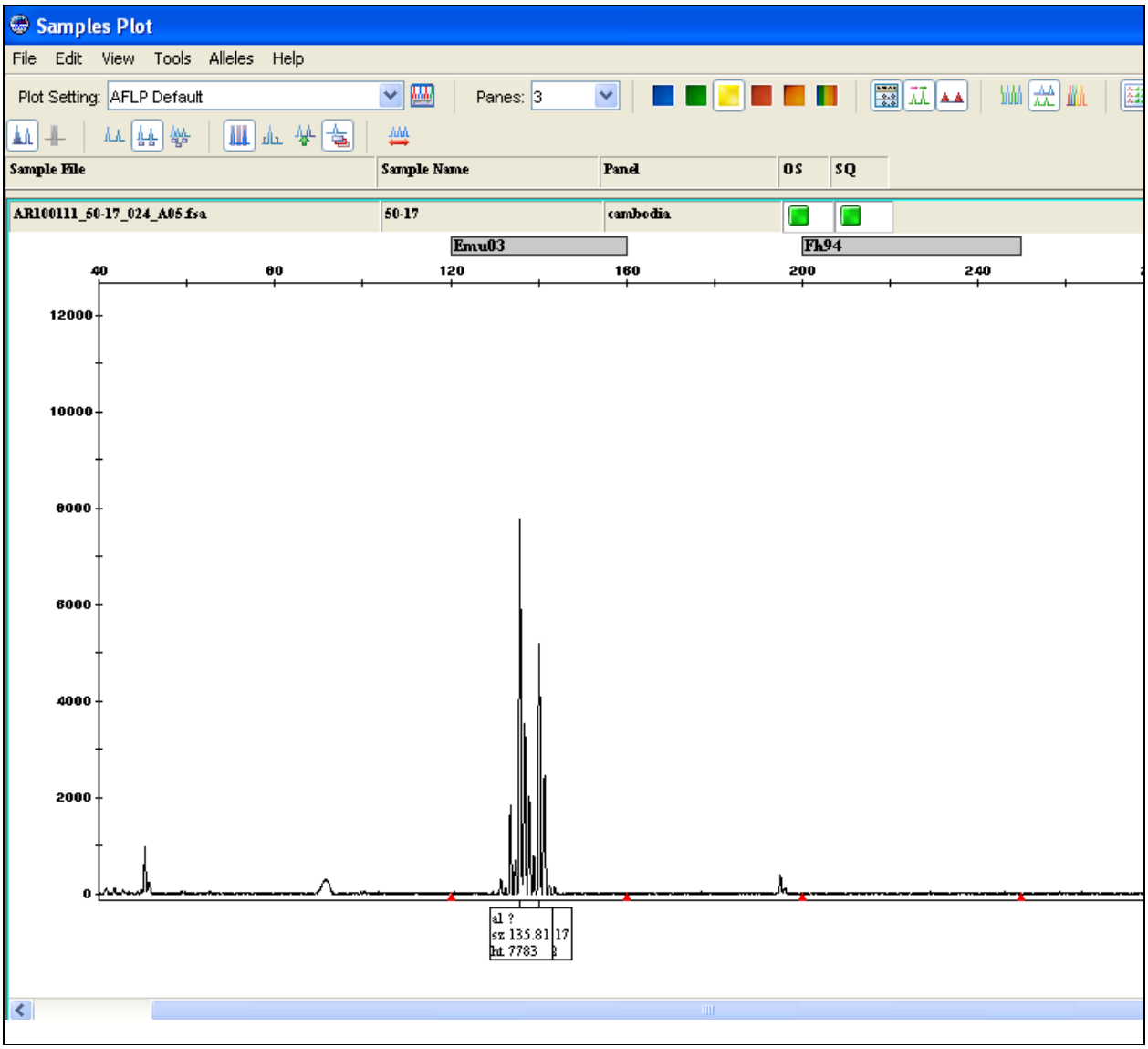

Figure 2. Electropherogram of a sample in which locus EMU03 was amplified.

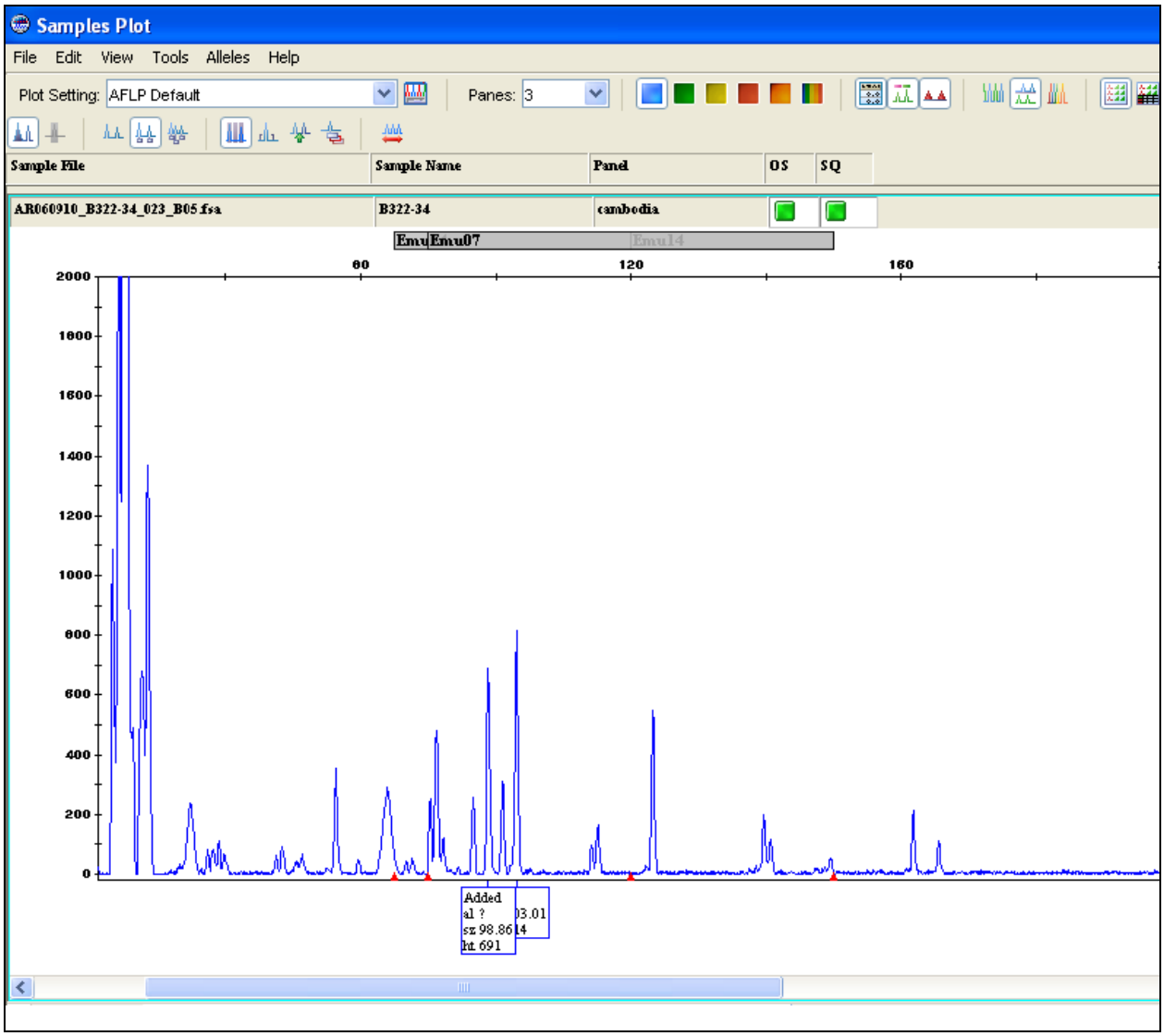

Figure 3. Electropherogram of a sample in which locus EMU04 was amplified.

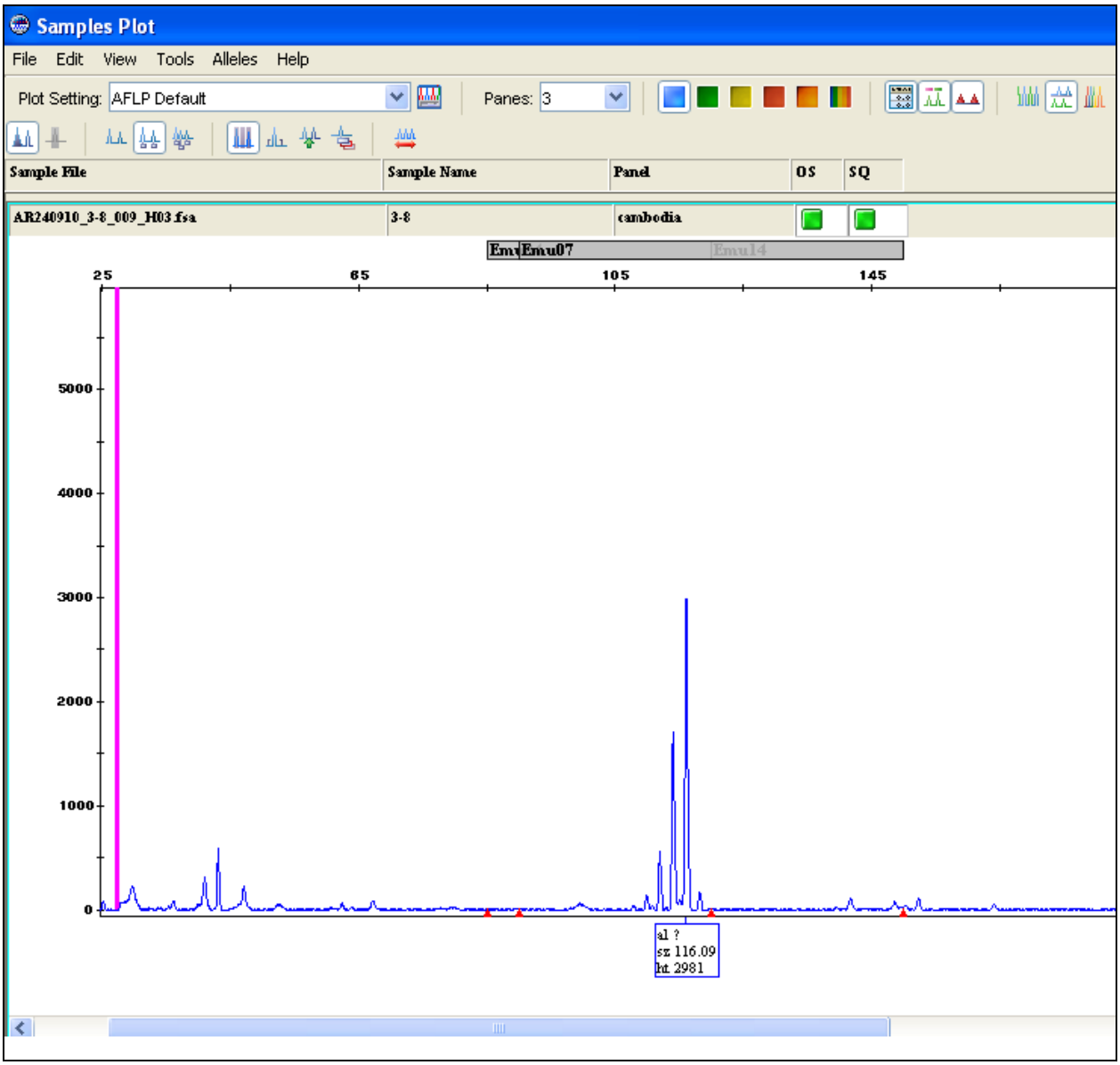

Figure 4. Electropherogram of a sample in which locus EMU07 was amplified.

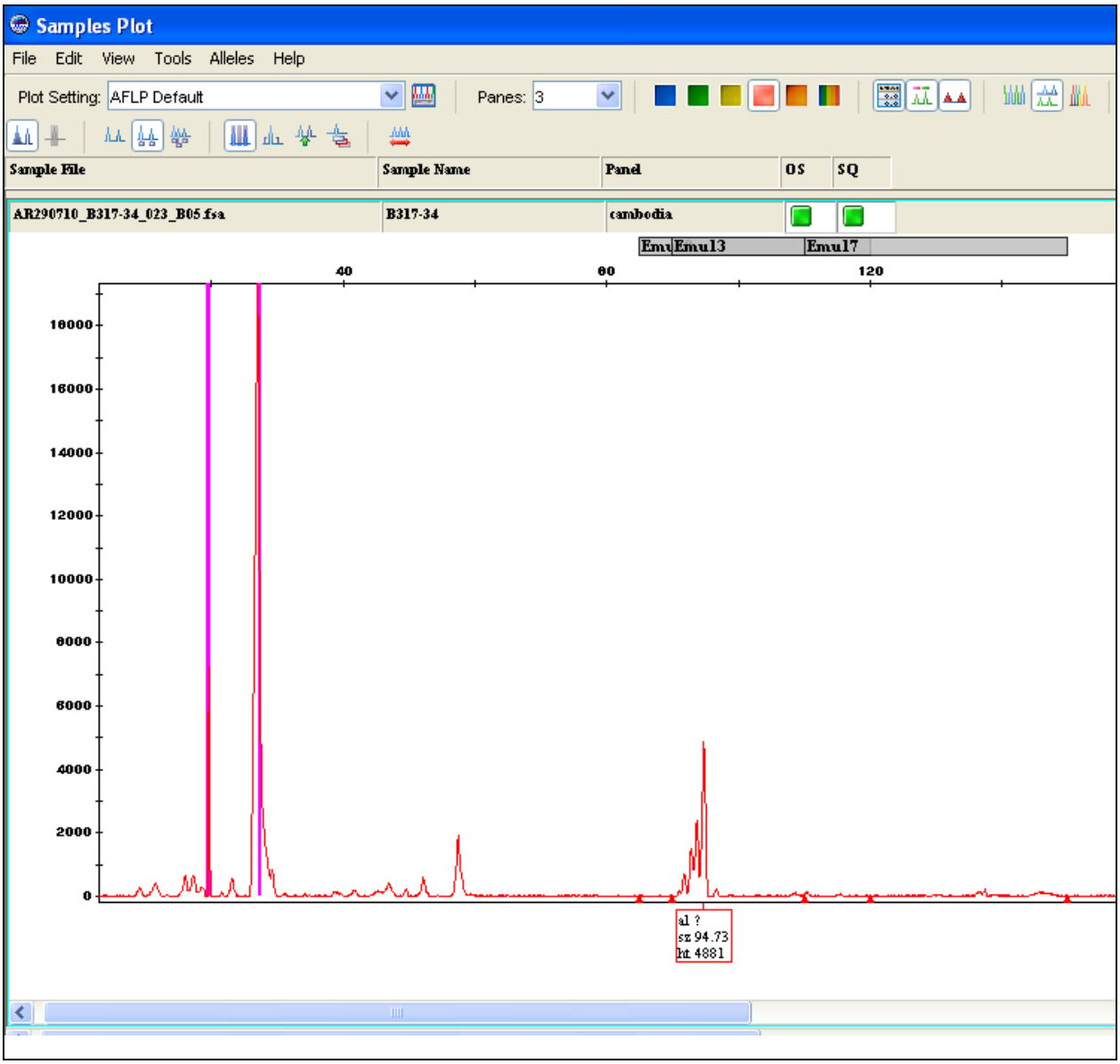

Figure 5. Electropherogram of a sample in which locus EMU10 was amplified.

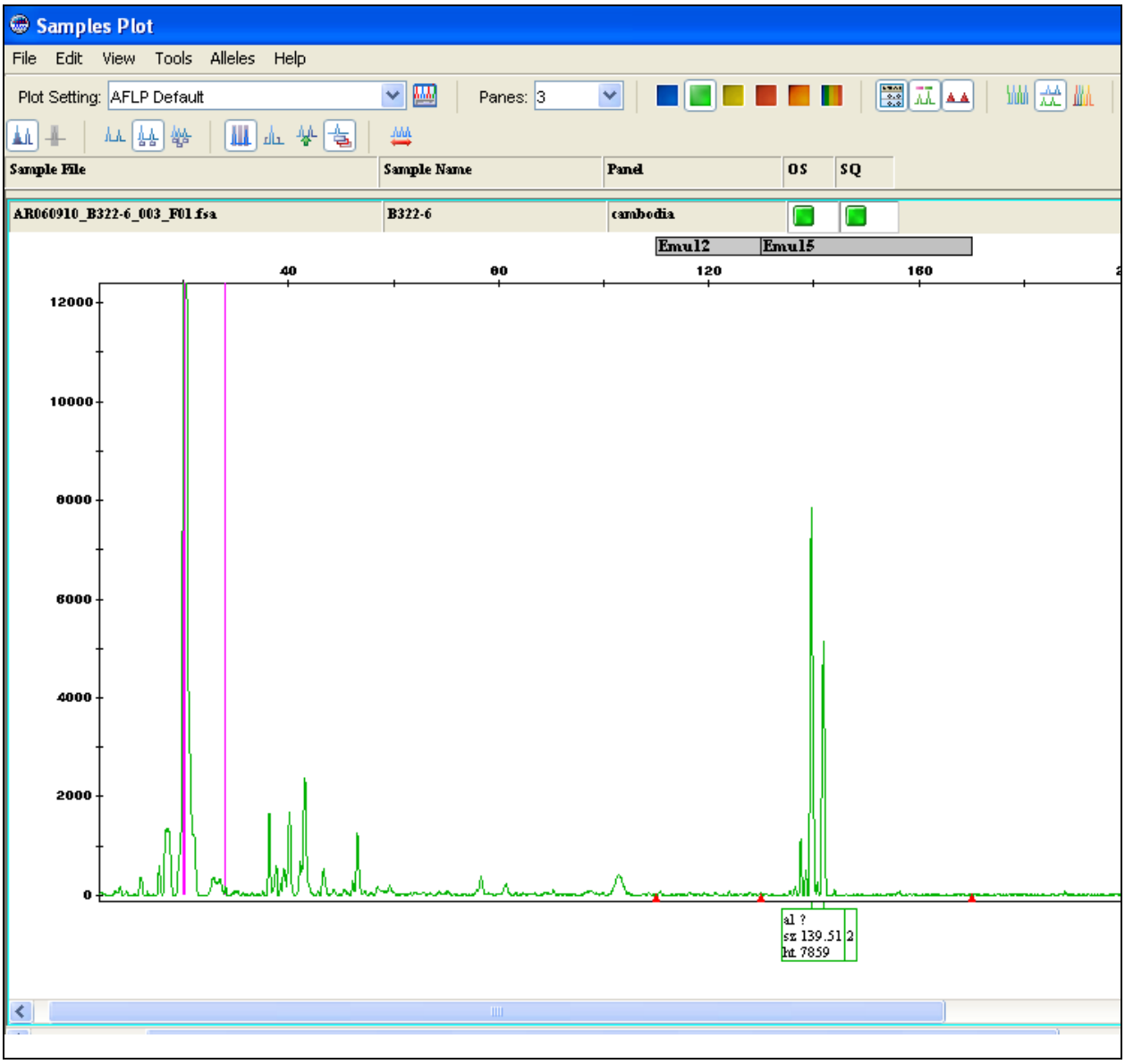

Figure 6. Electropherogram of a sample in which locus EMU12 was amplified.

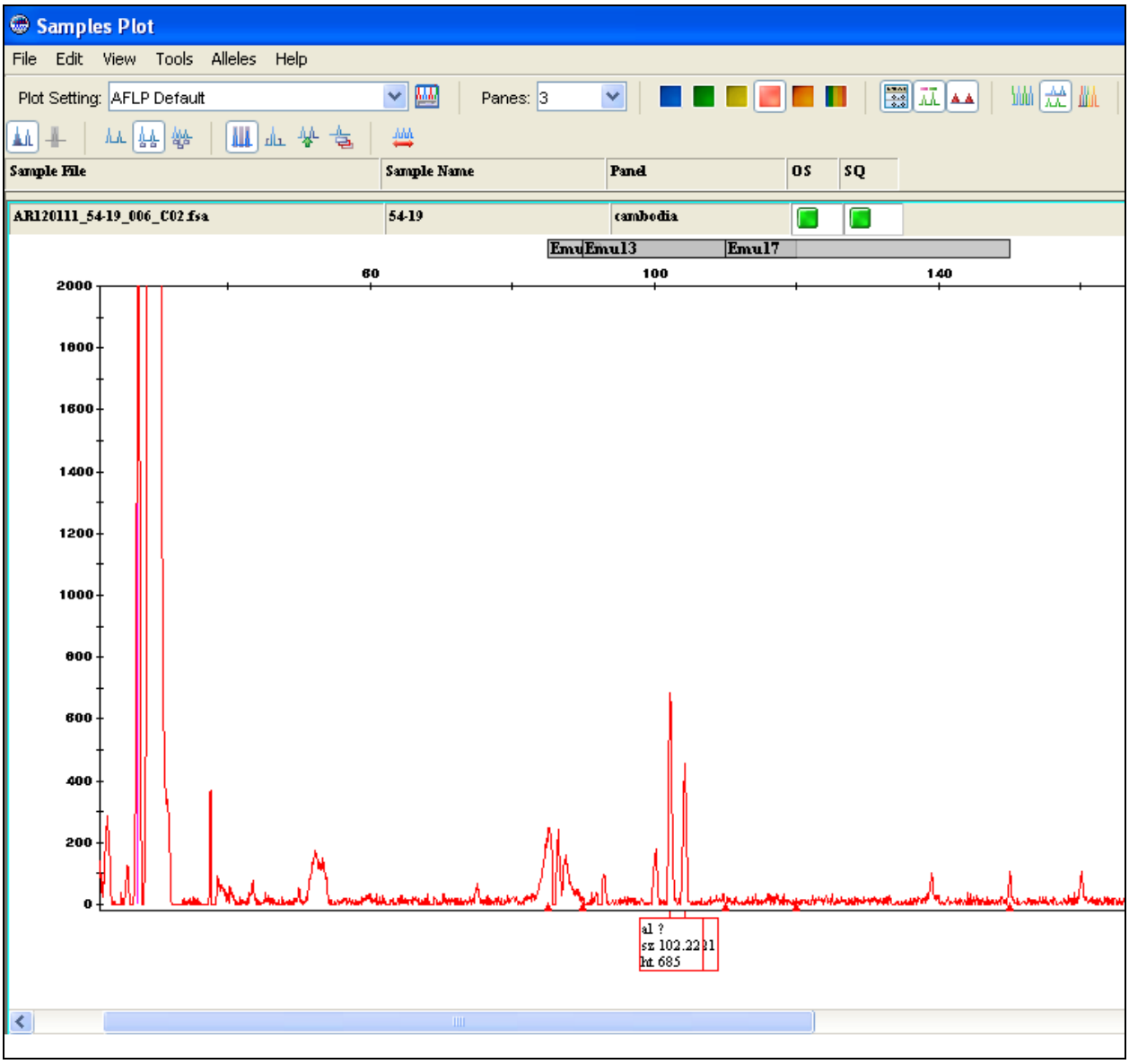

Figure 7. Electropherogram of a sample in which locus EMU13 was amplified.

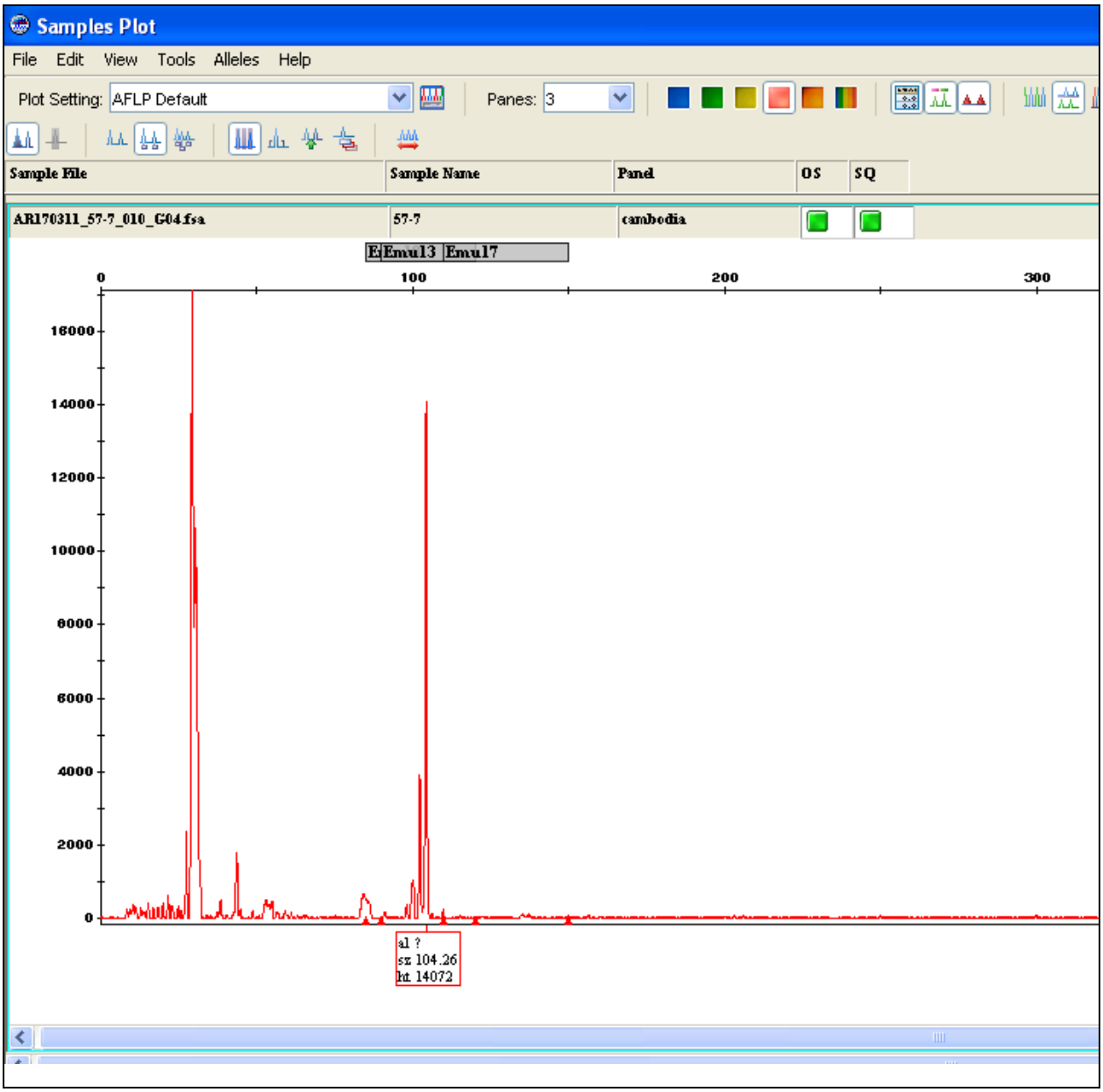

Figure 8. Electropherogram of a positive control in which locus EMU13 was amplified.

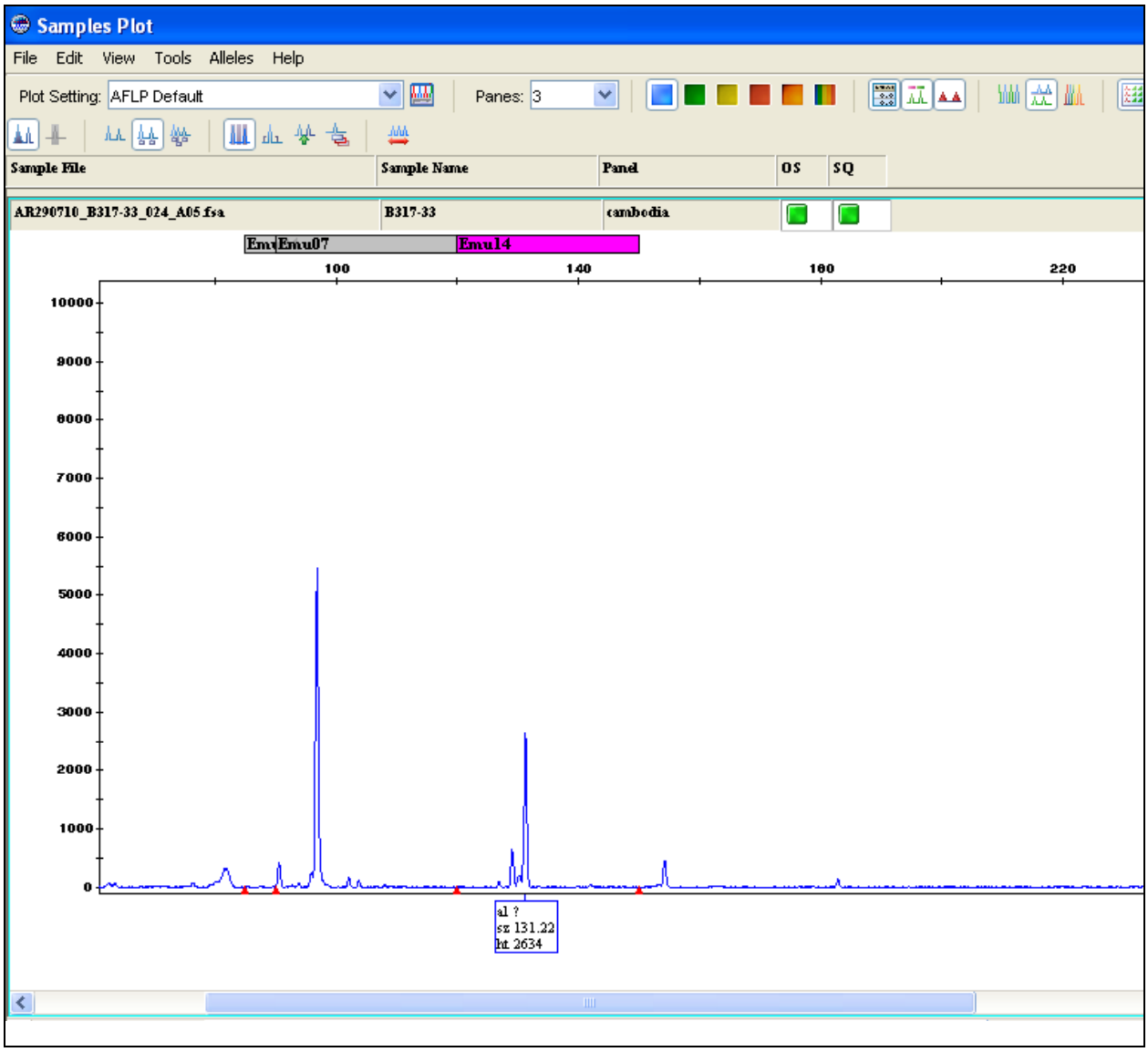

Figure 9. Electropherogram of a sample in which locus EMU14 was amplified.

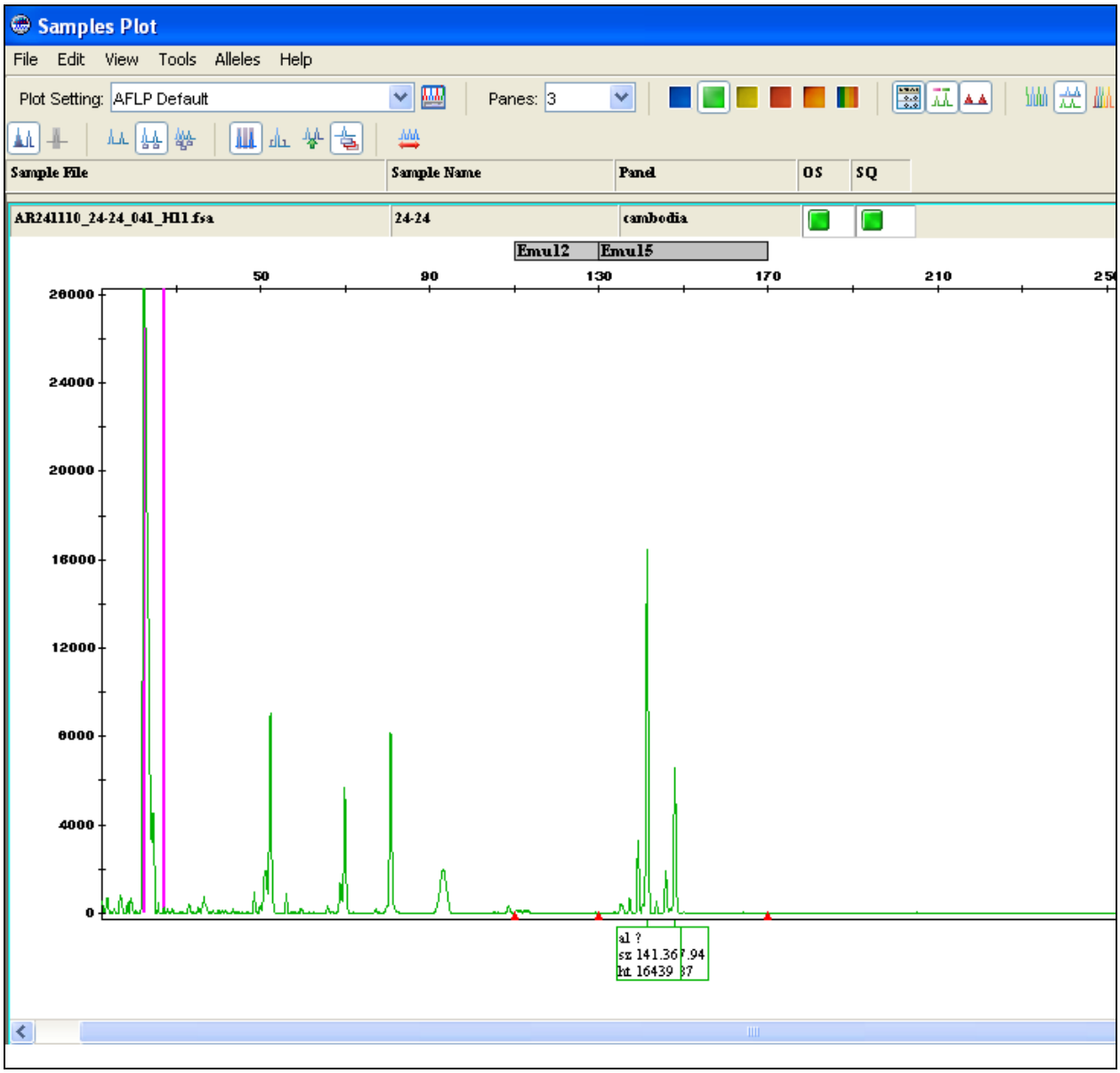

Figure 10. Electropherogram of a sample in which locus EMU15 was amplified.

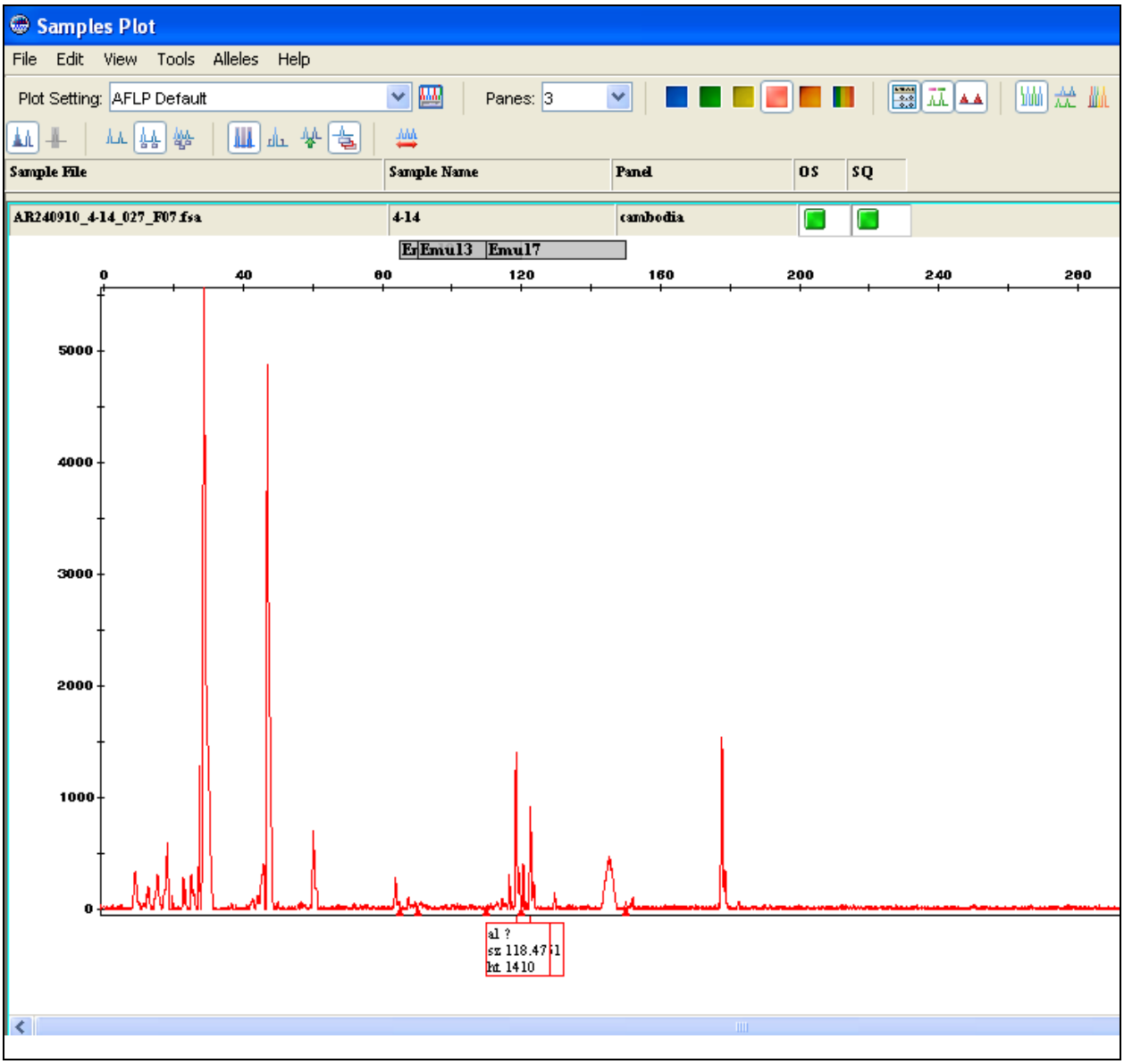

Figure 11. Electropherogram of a sample in which locus EMU17 was amplified.

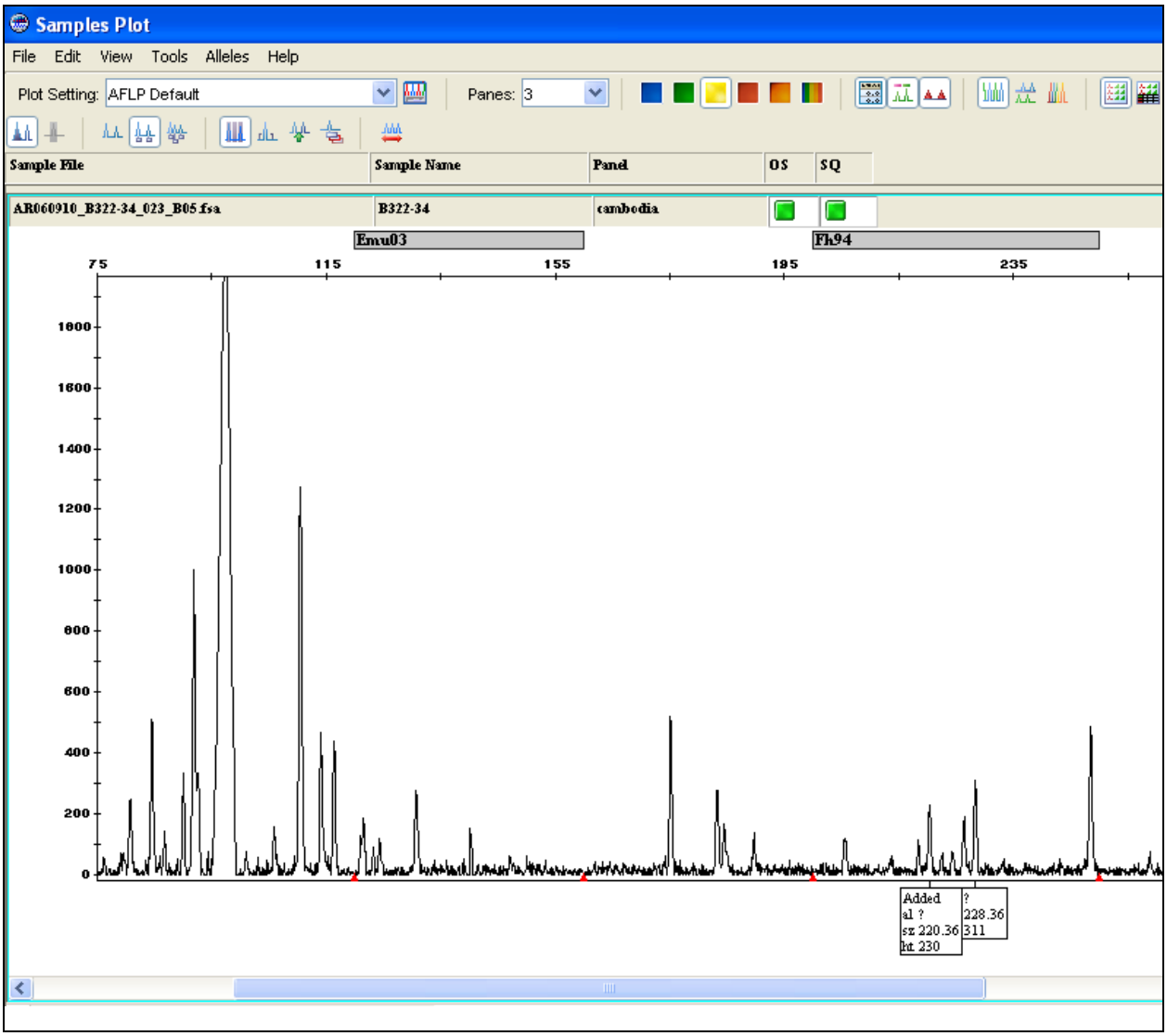

Figure 12. Electropherogram of a sample in which locus FH94 was amplified.

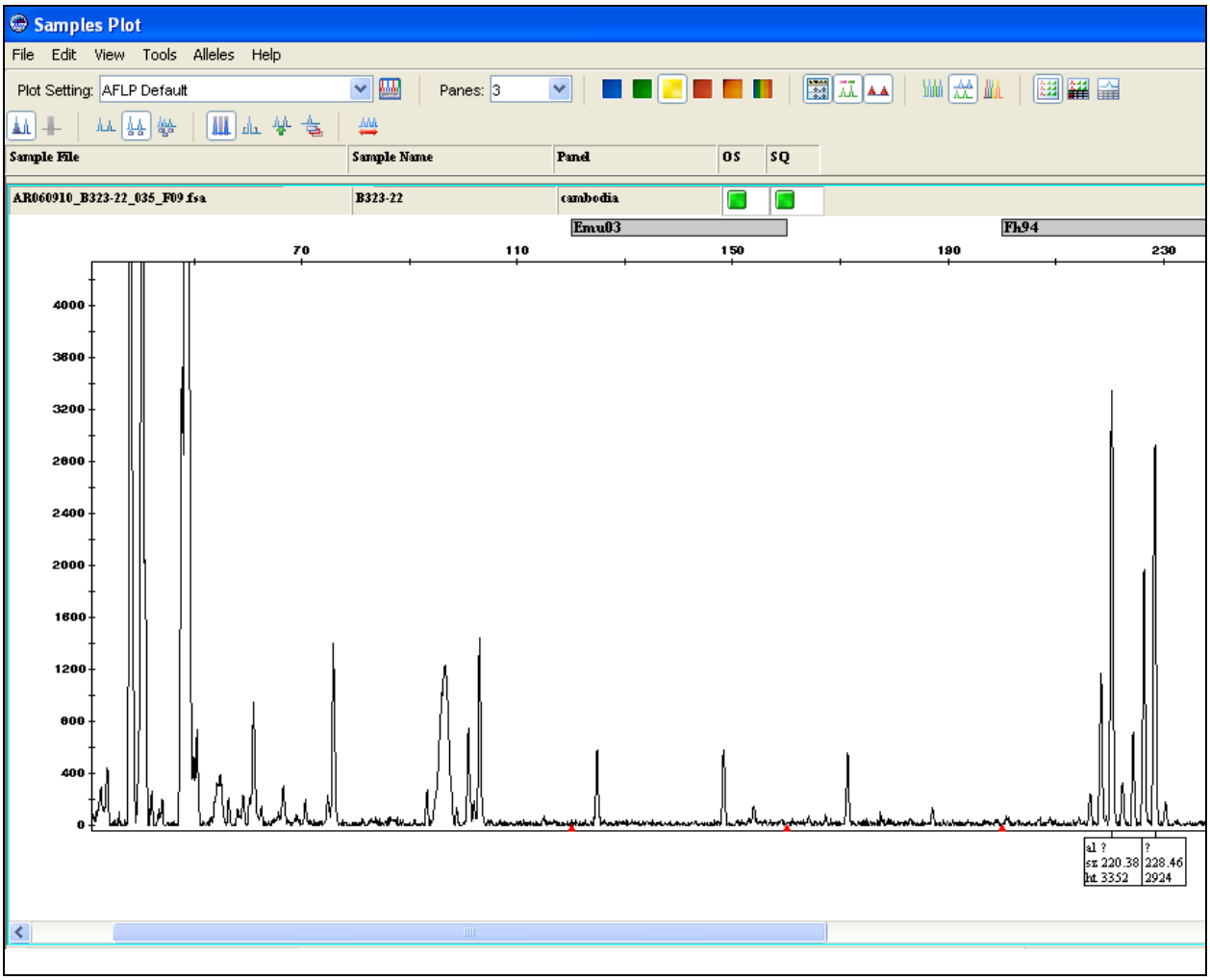

Figure 13. Electropherogram of another sample in which locus FH94 was amplified.

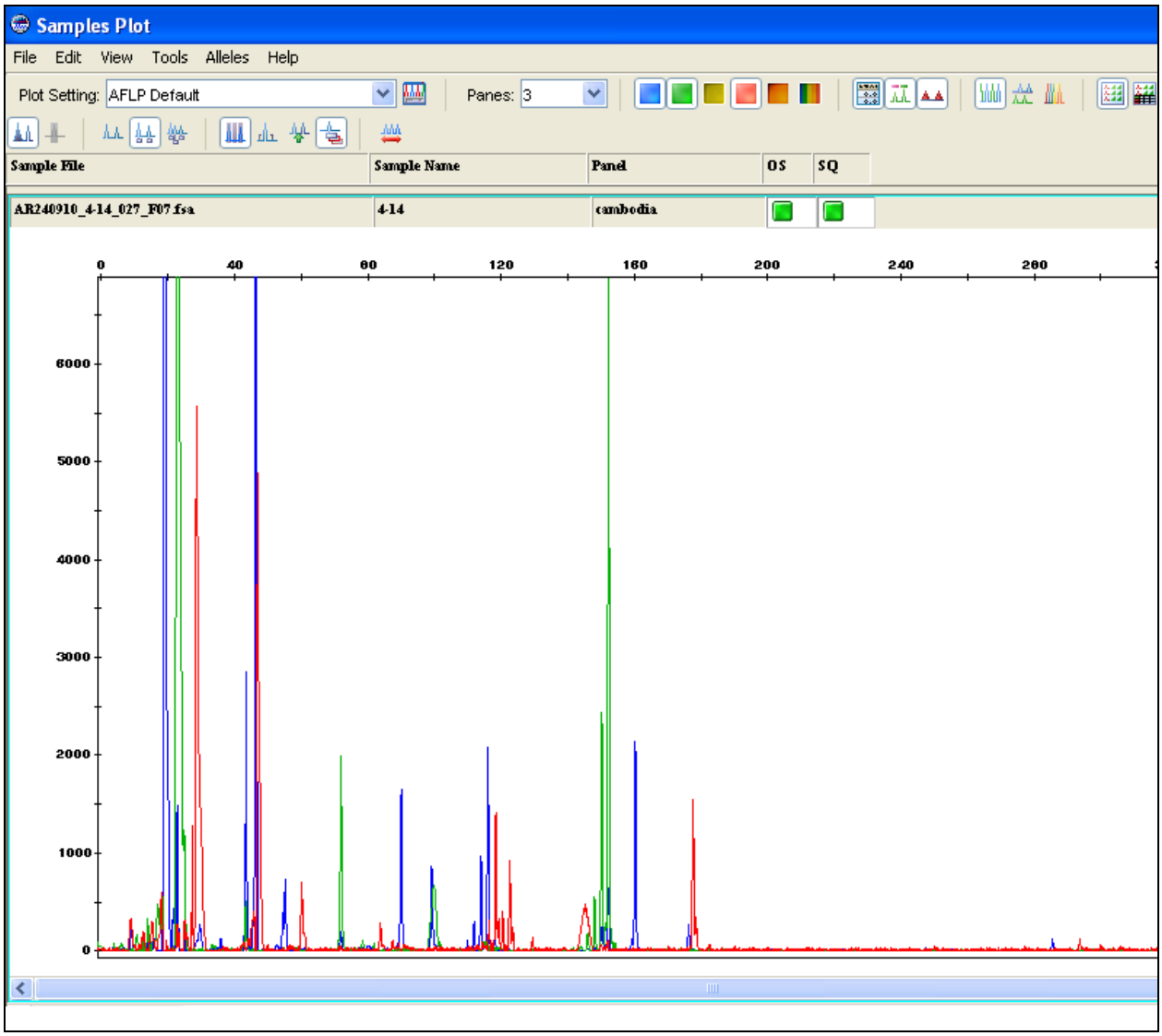

Figure 14. An example of what the electropherograms of a sample looks like before each locus is individually scored. This panel contained loci EMU07 (in blue), EMU17 (in red), and EMU15 (in green).

After all the genotypes were scored, we had a dataset of multiple genotypes (from the PCR repeats) per locus per sample. In some cases, none of the PCRs had worked at any locus, in which case, those samples could not be used. Some samples had genotypes at some loci but not at others, in which case the other loci were repeated and either worked or did not. Genotypes at each locus were classified depending on their quality as follows:

1. Some samples showed multiple (at least two) repeats of the same genotype and therefore, such samples had a tentatively confirmed genotype for that locus. Cells of this type were left uncoloured in the excel sheet.
2. Some samples showed a heterozygous genotype from at least one PCR amplification and homozygous genotypes from other PCRs. However, the homozygous genotypes were consistent with the heterozygous genotype, indicating allelic dropout rather than other laboratory error. For instance, the genotypes in three PCR repeats of a locus at the same sample could be 135/137, 135/135, and 137/137 or 135/137, 135/137, 137/137. These samples were assigned the heterozygous genotype, which would be the tentatively confirmed genotype, and coloured yellow.
3. Some samples showed genotypes in which one allele was repeatedly obtained but the second allele was not clear. For instance, one could find the genotypes 135/137, 135/139, 135/135, and 135/?. This would happen especially in samples that consistently gave peaks of low or very low intensity. In such cases, the genotype assigned included only the one consistent allele, 135/0 in the example above, and these cells were coloured light yellow.
4. Some samples consistently showed two different homozygous genotypes, indicating that there was probably allelic dropout in each situation rather than other laboratory error. For instance, one could obtain the genotypes 135/135, 137/137, 135/135, and 137/137. In such cases, the heterozygote genotype 135/137 was assigned, but the cells were coloured blue.
5. There were samples for which we could obtain only one successful PCR amplification. For instance, the genotypes could be 135/135, 0/0, 0/0, and 0/0, or 135/137, 0/0, and 0/0, or 135/0, 0/0, 0/0, and 0/0. These samples, for which we did not have a repeat reaction to confirm the genotype, were coloured pink. In the third example above, we scored genotypes of the type 135/0 if the peak 135 was of very low intensity and we, therefore, suspected that there might also be another allele that had not amplified. If samples had very low intensity peaks that were not clear, they were just scored as not having worked (0/0) and transferred to the next category below.
6. If the sample had not shown any amplification at the locus, its genotype was 0/0 and such cells were coloured grey.

Of the six categories above, the first three were categorized as genotypes of good quality (since there were confirmed genotypes/alleles) and the next two as genotypes of not-so-good quality. Datasets were then made with genotypes of only good quality (in which the not-so-good quality genotypes were scored as not having worked at all) and of good and not-so-good quality genotypes. Only samples that had genotypes from at least five of the nine loci (since EMU13

was excluded) were included. This led to 146 samples in the former dataset and 198 in the latter. The remaining 100 samples did not have even five loci that worked to any extent (even not-so-good data).

Using the Excel Microsatellite Toolkit (Park 2001), we checked for duplications in genotypes between samples in the good quality dataset. With the criterion that up to two alleles do not match, 456 matching pairs were found. This criterion was used since it is not uncommon, with dung-extracted DNA, to have allelic dropout during PCRs. Therefore, the genotype that is obtained may not be the correct genotype, even if there are 2-3 consistent repeats. However, 456 is an upper limit and the actual number of matching pairs was likely to be much smaller. This is because, when a two allele mismatch is allowed, the software does not discriminate between two allele mismatches at two different loci and two allele mismatches at the same locus. For example, with a two allele mismatch being allowed, a sample with genotypes 232/232 and 145/147 at two loci and another sample with genotypes 232/234 and 145/145 may be considered by the software as the same individual. But samples with genotypes 232/234 and 145/147, and 234/236 and 147/149 may also be considered as the same individual by the software. While the calling of the first pair as identical is justified as each individual may have shown allelic dropout at one locus each, the calling of the second pair as identical is not likely to be correct as these are two different heterozygotes. Thus, identification of matching genotypes using an automatic cutoff by the software assumes that the probability of making any type of error is the same, which is not true in practice. It is more probable that heterozygotes are wrongly genotyped as homozygotes because of allelic dropout than heterozygotes being wrongly genotyped as different heterozygotes across repeat PCRs. Therefore, once the set of matching pairs was obtained from the software, each of the pairs was manually examined to find out if the pairs could actually be the same because of allelic dropout or if they were different heterozygotes confirmed by repeated PCRs. Once this was done across loci, the number of matches dropped drastically. After this set of checks, the not-so-good data were also included to see if the samples that matched based on only loci with good quality genotypes remained identical or became different when the loci with not-so-good genotypes were also included. Since the not-so-good genotypes may not be accurate data, allowances were made for mismatches in these, and a further two (or, occasionally, three depending on the type of error) allele difference at these data were tolerated as being consistent with the same genotype. Therefore, if the assignment of matches between pairs of individuals is

biased, it is biased towards a greater number of matches and hence a smaller number of unique individuals. In previous studies using dung extracted DNA, differences in one or two loci were used as the cutoff to identify matching individuals in an African elephant population (Eggert *et al*. 2007), and samples with four or fewer alleles different were reexamined and those with up to two different alleles and the same sex were considered the same individual in an Asian elephant population (Ahlering *et al*. 2011).Based on all the above checks, a total of 194 individuals' genotypes were usable, and these corresponded to 112 unique genotypes.

Appendix 2: Allele frequencies at different loci.

Locus	n	Allele	Frequency
EMU03	192	132	0.005
		134	0.432
		136	0.089
		138	0.063
		140	0.411
EMU04	198	97	0.015
		99	0.308
		101	0.010
		103	0.657
		105	0.010
EMU07	176	106	0.006
		108	0.097
		110	0.199
		112	0.017
		114	0.142
		116	0.528
		124	0.011
EMU10	204	94	0.608
		96	0.225
		100	0.039
		102	0.059
		104	0.054
		106	0.015
EMU12	200	139	0.520
		141	0.445
		148	0.005
		152	0.025
		158	0.005
EMU14	202	127	0.040
		129	0.223
		131	0.455
		133	0.084
		137	0.168
		139	0.025
		141	0.005
EMU15	192	142	0.156

		146	0.026
		148	0.042
		150	0.047
		152	0.531
		154	0.198
EMU17	164	118	0.244
		120	0.152
		122	0.207
		124	0.146
		126	0.091
		128	0.146
		134	0.012
FH94	174	215	0.006
		217	0.184
		221	0.207
		223	0.155
		225	0.092
		227	0.121
		229	0.236

Appendix 3: Comparisons of model output (AICc scores, Akaike weights and population estimates) between standard CMR models and Lukacs & Burnham (2005-b) mis-identification models in software MARK.

Model	AICc	AICc Weights	N ± SE
M0	-117.5	0.44	135 ± 18
M0 mis-id	-116.0	0.21	98 ± 39
Mh	-115.4	0.16	136 ± 18
Mh mis-id	-114.0	0.08	98 ± 39
Mt	-114.0	0.08	135 ± 18
Mt mis-id	-111.9	0.02	135 ± 18
Mht	-108.7	<0.01	143 ± 29
Mht mis-id	-107.2	<0.01	86 ± 44

Appendix 4: Modeling output from continuous occasion CMR models in software CAPWIRE for estimating Asian elephant population size in PPWS.

Log L TIRM	Log L ECM	Selected Model	α	N^a	N^b
-603.2	-646.3	TIRM	5.9	32	122

Log L Log likelihood scores for the two innate rates (TIRM) and the even capture models (ECM)

α Relative capture probability of type A individuals *vs* type B individuals

N^a Mean estimate of number of type A individuals in the population

N^b Mean estimate of number of type B individuals in the population