The Rufford Small Grants Foundation

Final Report

Congratulations on the completion of your project that was supported by The Rufford Small Grants Foundation.

We ask all grant recipients to complete a Final Report Form that helps us to gauge the success of our grant giving. We understand that projects often do not follow the predicted course but knowledge of your experiences is valuable to us and others who may be undertaking similar work. Please be as honest as you can in answering the questions – remember that negative experiences are just as valuable as positive ones if they help others to learn from them.

Please complete the form in English and be as clear and concise as you can. We will ask for further information if required. If you have any other materials produced by the project, particularly a few relevant photographs, please send these to us separately.

Please submit your final report to jane@rufford.org.

Thank you for your help.

Josh Cole, Grants Director

Grant Recipient Details	
Your name	Worata Klinsawat
Project title	The effect of habitat fragmentation on the genetic structure and gene flow of Indochinese tigers (*Panthera tigris*) and elephants (*Elephas maximas*), TH
RSG reference	9926-1
Reporting period	2012
Amount of grant	£ 6,000
Your email address	worata@gmail.com
Date of this report	2013-06-13

1. Please indicate the level of achievement of the project's original objectives and include any relevant comments on factors affecting this.

Objective	Not achieved	Partially achieved	Fully achieved	Comments
Build local capacity for landscape genetic monitoring, wildlife forensics, and national wildlife genetic database			Yes	Patrol rangers, NGO partners and local students in our systematic sampling teams are now well trained for technical skills necessary for landscape genetic sampling. Government agencies have a better understanding about the significance to incorporate genetic information into ecological and demographic monitoring. We also successfully promote research and education in conservation genetics at national and international level.
Assessing the genetic structure and patterns of gene flow of the tiger and elephant populations across Tennassarim Range (WEFCOM: Western Forest Complex, KKFC: Kaeng Krachan Forest Complex), Thailand.		Yes		We successfully established a national genetic database of the threatened species. Based on mitochondrial DNA (mtDNA), we identified three haplotypes of wild tigers in Thailand. The newly discovered WEFCOM haplotype is unique to the wild tigers from WEFCOM and serve as the intermediate link to Siberian and Indochinese tiger in evolutionary history. We detect no landscape barrier against gene flow within WEFCOM for both tiger and elephant. However, between WEFCOM and KKFC, we have difficulties in obtaining faecal sample in some rugged terrain. We are in the process of completing gene flow analysis of the elephant populations between these two isolated habitat patches. Similar to tiger diversity, we also discover new elephant haplotypes unique to WEFCOM area. After completing the population structure and gene flow analysis, we will form recommendations about effective conservation practices for tiger and elephant as well as publish our finding in peer-reviewed journal in 2015.

2. Please explain any unforeseen difficulties that arose during the project and how these were tackled (if relevant).

2.1 Difficulty in obtaining fresh fecal samples in some sampling sites

The quality of the faecal DNA can be greatly improved by sampling fresh feces (<2 days) to avoid

exposure to the UV light and moisture. These two factors strongly degrade DNA and inhibit PCR amplification. Good DNA quality and quantity lead to reliable microsatellite genotypes critical for inferring population connectivity across the landscape. Although we have made great sampling efforts, obtaining fresh tiger and elephant faeces are one of the challenges in this project. In some areas, for example, the rugged terrain in the northern part of Kaeng Krachan Forest Complex (KKFC) is only accessible by inflatable boat. Although we spent two weeks in such area, we found no tiger scats and only four old elephant dungs. Across the whole KKFC, no tiger scats were found, but a few tracks were observed. In addition, we found no elephant samples and seven old tiger scats at the Western Thung Yai Wildlife Sanctuary. We are in the process of optimizing multiplex genotyping techniques to achieve reliable genotypes out of those old scats. These protocols require more chemicals and are more time consuming than regular methods applied to fresh samples. However, we gradually observe some improvement and other conservation genetic study can adopt our protocol.

2.2 Limitations inherent to noninvasive samples: low DNA quality and quantity

Poor quality results from the presence of exogenous DNA from plants, bacteria and/or prey species, all of which lower genotyping success rate. Moreover, harsh environmental conditions in the tropics also further degrade DNA. Previous studies have shown that high atmospheric moisture, UV intensity and temperature are the main factors degrading DNA into short fragments. To solve this problem, we used PCR primers (Luo *et al.* 2004, Driscoll *et al.* 2009) to amplify short fragment of mitochondrial DNA (mtDNA, <200-300 bp). However, the total amount of targeted DNA in faeces can be very low, particularly the nuclear microsatellite, resulting in genotyping errors (Figure 1). These errors include allelic dropout (only one allele of a heterozygous individual is amplified, producing false homozygous genotypes), false allele amplification (amplification artifacts misinterpreted as true allele), and null alleles (no amplification of any allele).

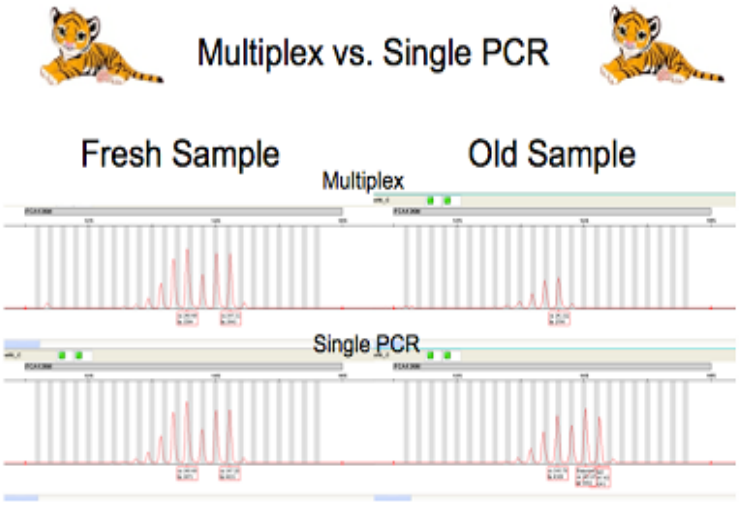

Figure 1*: Multiplex vs. Single PCR approach. Using fresh samples, genotyping error such as allelic dropout is low in both multiplex and single PCR. Both alleles (141, 147 bp) were amplified. However, allelic dropout, usually non-amplification of the larger-size allele, becomes dominant in old sample (> 2d). Multiplex approach failed to amplify the allele size of 147 bp, whereas single PCR yielded both 141-bp and 147-bp alleles.*

To obtain accurate genotypes, we conducted the maximum of nine replicates of the same fecal samples for each microsatellite locus (multiple-tube approach: Taberlet et al. 1996). We also tackled PCR challenges using multiplex PCR method which co-amplifies several microsatellite loci at the same time, allowing more efficient use of faecal DNA. Although multiplex system worked well with the pilot study based on blood sample of tiger and elephant, the PCR success rates varied when applied to faecal samples, particularly old samples (>2 days). Often time, we firstly applied multiplex system then followed by different dilutions of the PCR products and then re-amplified using single-

locus PCR. This multiplex-single PCR approach required time-consuming and chemical adjustment. However, it increased the amount of faecal DNA template and reduced genotyping errors. In short, most genotyping difficulties can be overcome using rigorous methods which require more time and expenses. Our PCR success rates in our study were moderate for tiger (67%) and high for elephant (80%). It is not surprising that the success rate of elephant was higher than that of tiger due to the diet content. Being herbivore, the PCR inhibitors are microbes and secondary compounds in plants. Tiger scats, in contrast, contain both microbe and prey DNA which strongly interferes with PCR amplification.

3. Briefly describe the three most important outcomes of your project.

3.1 Built local capacities for landscape genetics

In Thailand, capacity for genetic monitoring to promote the species recovery and survival in both contiguous and human-dominated landscape is still lacking. Assessing population abundance and spatial genetic structure of the elusive, wide-ranging species are the conservation needs. To achieve such goals, firstly, we formed a network of committed individuals and systematic teams of patrol rangers, NGOs partners (WCS/WWF/FREELAND Foundation), and local students for landscape-scale non-invasive sampling. After lectures and training activities, our teams have acquired technical skills including the abilities to differentiate between tiger and leopard scats, scat age, and appropriate amount of scat surface collected (Figure 2). These skills are critical to obtain good quantity and quality of fecal DNA, improving results of downstream analyses (microsatellite genotyping). Our teams will be able to apply the practical sampling method and sharing knowledge necessary for future landscape genetic sampling, for example, DNA-based mark-recapture survey, behavioral study of paternity, and disease surveillance.

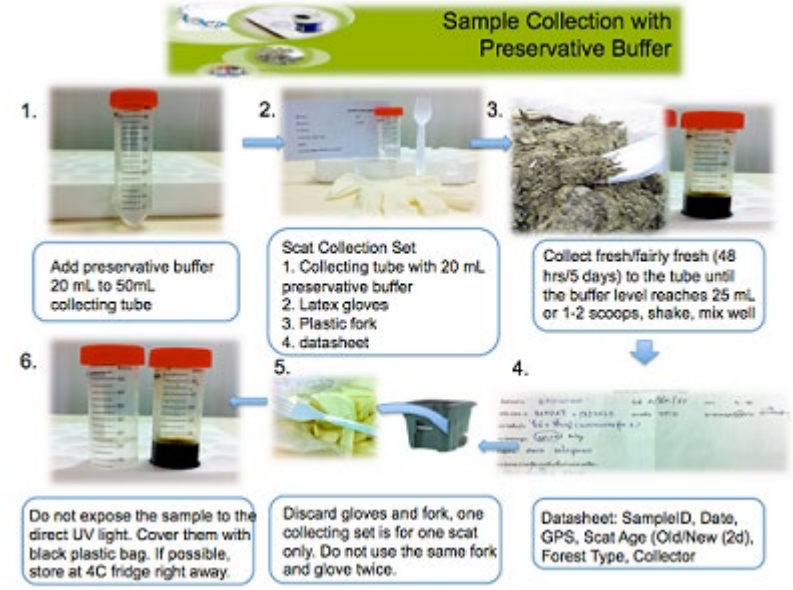

***Figure 2**: Fecal Sample Collection Protocol based on DMSO preservative buffer. We created posters and distributed this protocol to patrol rangers, park staffs, our NGO partners as well as government agencies.*

During the workshops, we emphasized the integration of genetic monitoring into the ongoing ecological monitoring such as camera trapping and radio telemetry. Analyses of such comprehensive data will aid in understanding the evolutionary history and consequently improve conservation planning. Via lectures and training activities, we also underscored the importance of maintaining genetic diversity in order to retain the populations' genetic health which is the ability to adapt to the changing environment.

We also promoted research and education in conservation genetics at national and international level. We have trained undergraduate students from local universities from the Faculty of Forestry and the Faculty of Veterinary Medicine from Kasetsart University and School of Bioresources and Technology from King Mongkut's University of Technology Thonburi. Extending our networks of young and dedicated conservationists overseas, we offered summer research experiences to two undergraduate students from University of Minnesota, U.S.A. and Macquarie University, Australia. In addition to wide range of technical skills in the field and in laboratory, we provided phylogenetic computer training. These skills enabled the students to assess NCBI resources (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov), construct phylogenetic relationships, and analyze preliminary data of tiger and elephant mtDNA diversity. We believe that, these students have gain more confidence to pursue their passion in conservation genetic-related profession.

3.2 Established national genetic database to support conservation genetic monitoring, discovered new tiger haplotype unique to WEFCOM habitat, and develop robust DNA-based forensic approaches to strengthen law enforcement in illegal wildlife trade.

Advances in high-throughout sequencing and population genomics have improved precision in analyzing large quantity of data relevant to conservation of endangered species. Collaborated with graduate students from Faculty of Veterinary Medicine, Kasetsart University, NGO partners, and government agencies, we are in the first phase of establishing genetic database of Thailand's threatened species both in the wild and captivity. Our aim is to promote the use of genetic information to better understand evolutionary history and strengthen conservation management of the threaten species in Indochinese and Sundaic zoological zones.

This database and our cost-effective forensic technique will aid in law enforcement of illegal wildlife trade by tracing the species and its geological origin. Thailand is one of the major transit hubs for wildlife trafficking including ivory, tigers and their parts from Southeast Asia to China. Based on our mtDNA diversity result, the newly discovered WEFCOM haplotype is only found in the wild tigers from WEFCOM (Figure 3). The captive populations across Thailand do not harbor this haplotype. Therefore, if we detect WEFCOM haplotype in confiscated tiger and their products, we can be certain of their origin from WEFCOM habitat.

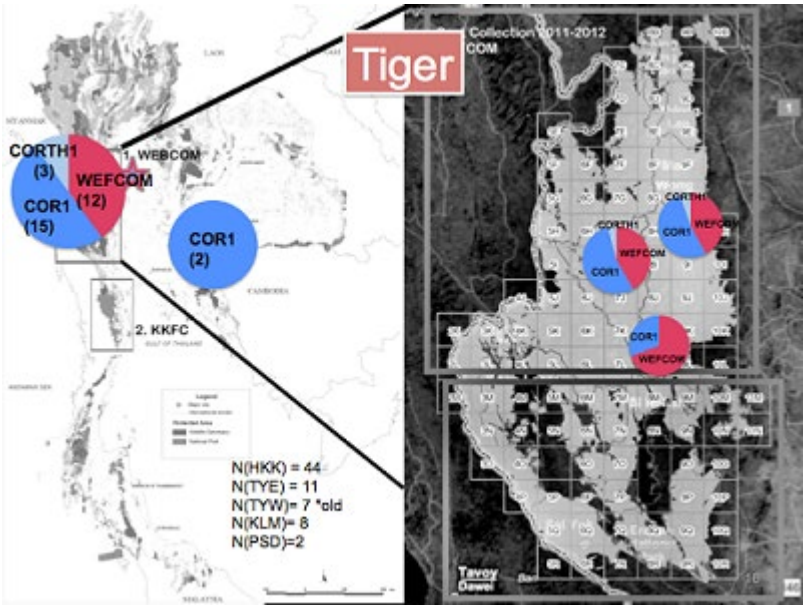

Figure 3: Haplotype Distribution. *The newly discovered WEFCOM haplotypes is shown in red, COR1 in blue, and CORTH1 in light blue. The numbers in parenthesis indicated the number of tiger individuals. We found no scats in the KKFC. The map on the right is modified from WCS occupancy survey.*

In terms of evolutionary significance, this WEFCOM haplotype is the missing link between the Indochinese tiger (*P. t. corbetti* I) and Siberian tiger (*P. t. altaica*). In contrast to the hypnosis of eastward expansion from Central Asia to Russian Far East (Driscoll *et al.* 2009), our result supports the northward expansion. Based on the phylogenetic relationships (Figure 5), WEFCOM and Siberian tigers are closely related to each other. This result supported that the modern Siberian tigers were established by dispersal of tigers from the mainland Indochina to Mongolian steppe, Ruffian Far East and Central Asia in the last ten thousand years (Kitchener 1999; Kitchener and Dugmore 2000; Luo *et al.* 2004).

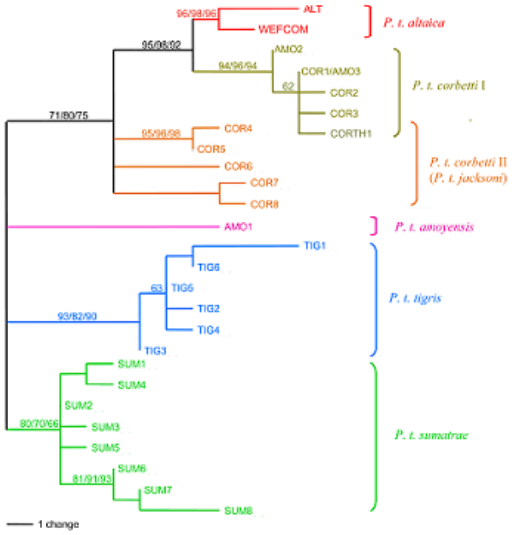

Figure 4. *Phylogenetic tree based on our concatenated mtDNA sequences (2,621 bp) and reference sequences from Luo et al. 2004 (4,078 bp, Maximum Parsimony). Our newly discovered WEFCOM haplotypes was grouped together (high bootstrap value) with ALT haplotypes of Siberian tiger (P. t. altaica) as shown in red. The other new haplotypes (CORTH1) was clustered within the clade of Indochinese tigers (P. t. corbetti I) as shown in yellow.*

Overall, Thailand harbours three tiger haplotypes: WEFCOM, COR1, and CORTH1 based on 72 tiger scats and 4078-bp mtDNA sequences (Figures 4 and 5). COR1 is the major haplotype found in our study from both western and eastern forest complexes as well as the rest of Southeast Asia. Due to only one haplotype detected in the eastern forest complexes, it is likely that this population is experiencing genetic bottleneck which is the reduction or depletion of genetic variation. However, our sampling from this area is very limited (three scats) due to smaller number of individuals compared to the western populations. More sampling is needed to address the issue of bottleneck and inbreeding level, allowing us to extrapolate viability of this population. CORTH1 is the new haplotype from WEFCOM habitat. Unlike WEFCOM haplotype, CORTH1 can be found in Thailand captive tigers. Based on the number of haplotypes, Indian tigers (32 haplotypes) retain the highest genetic diversity of the remaining tiger populations (Mondol *et al.* 2009) due to either higher historical effective population sizes or more diverse types of habitats.

3.3 Establish a panel of 14 polymorphic microsatellite loci for population structure analysis and developed robust molecular sexing technique.

Out of the screened 25 microsatellite loci from Luo *et al.* 2004, we established the panel of 14 polymorphic microsatellite markers suitable for genetic diversity and paternity test of tiger populations in Southeast Asia. We modified Zou Zhengting's multiplex PCR amplification, which was developed for tiger blood samples (Zhengting, personal communication), to amplify our faecal DNA. This multiplex method already incorporated sex identification, therefore, consuming less DNA template and less genotyping time. Population structure analysis based on microsatellite genotypes (Figure 6) indicated the substructure of potentially four family groups (K=4) of tigers in WEFCOM habitat. We found no landscape barriers which could prevent gene flow between Huai Kha Khaeng Wildlife Sanctuary in the eastern part of WEFCOM to Eastern Thuang Yai Wildlife Sanctuary in the

western part. The moderate levels of genetic diversity based on the average allelic richness, expected heterozygosity (H_e), observed heterozygosity (H_O) were shown in Table 1.

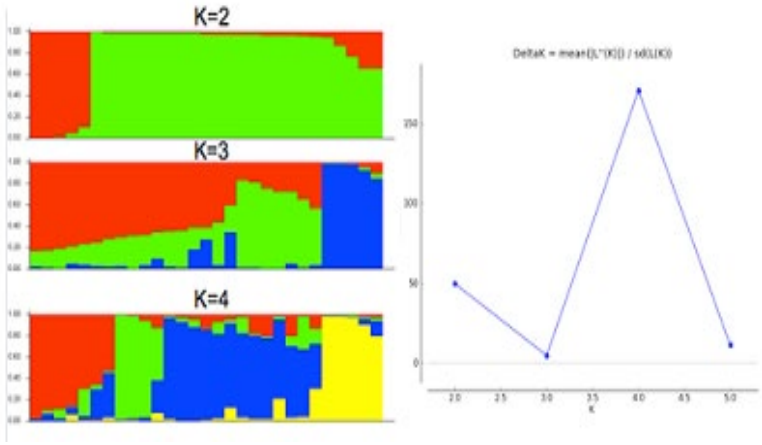

***Figure 5**: Population Structure analysis of wild tigers in WEFCOM, Thailand, based on 14 microsatellite genotypes. In the left histogram, three different numbers of clusters (K = 2, 3, and 4, posterior assignment probabilities) were implemented in STRUCTURE program. K = 4 was selected as the most likely cluster under the delta K comparison in the right graph.*

Table 1: Number of alleles, expected heterozygosity (He), observed heterozygosity (Ho) for 14 microsatellite loci

Locus	Number			
	Alleles	He	H0	PIC
FCA005	5	0.581	0.375	0.536
FCA008	7	0.779	0.500	0.728
FCA043	3	0.577	0.435	0.498
FCA069	7	0.762	0.643	0.705
FCA077	7	0.828	0.409	0.783
FCA091	7	0.820	0.381	0.771
FCA105	6	0.702	0.444	0.631
FCA126	6	0.743	0.808	0.686
FCA161	4	0.553	0.407	0.458
FCA211	5	0.635	0.333	0.580
FCA220	6	0.790	0.654	0.741
FCA304	7	0.758	0.360	0.711
FCA310	5	0.708	0.444	0.650
FCA441	6	0.816	0.393	0.772
Average (all)	5.79	0.718	0.471	
Std.Dev. (all)	1.25	0.026	0.027	

Estimation of sex ratio and sex-specific population genetic parameters facilitates the study of dispersal pattern, social systems, as well as, improves captive breeding management. When microsatellite genotype is not required and only sex information is needed, we developed reliable, inexpensive PCR method for tiger sex identification. We designed and optimized SRY primers to exclude amplification of prey DNA. The sex scoring was confirmed using the reported DBY primers. Using co-amplified DBY-SRY fragments and replicates, our technique yielded 100% success rate based on blood and fresh fecal samples and 59% success on old fecal samples. Our method improved the accuracy of molecular sexing by reducing allelic dropout errors and prey's DNA interference. We gave a poster presentation of this technique at FAVA conference (Federation of Asian Veterinary Associations) in Taipei, Taiwan during January 4-6, 2013 (Figure 7).

Figure 6. *Molecular sexing poster presented at FAVA Conference with Rufford logo.*

3.4 Discovered new elephant haplotypes and illustrated spatial haplotype distribution of elephant population in WEFCOM

Based on 181 elephant faecal samples and 600-bp mtDNA fragment in control region (primer MDL3/MDL5, Fernando and Lande, 2000), we identified 9 haplotypes, three of which are new haplotypes (BHa, Aha, AHb) unique to WEFCOM habitat (Figure 8). Similar to tiger forensic investigation, we can use this information to identify ivory's geographic origin. Compared to the WEFCOM populations, the KKFC population exhibited less level of genetic diversity potentially due to lower effective population sizes and the bottleneck effects. We made recommendations to the government agencies that conservation efforts should focus on maintaining these haplotypes to ensure the adaptive ability toward environmental changes. For structure analysis, the samples were genotyped at 10 microsatellite loci. These markers were the same panel used in genetic study of Asian elephants in Salakpra Wildlife Sanctuary, Thailand (Kongkrit *et al.* 2008) and Lao PDR (Ahlering *et al.* 2011). We are finalizing the genotyping result to indicate the degree of isolation between WEFCOM and KKFC, assessing the effect of human settlement toward elephant dispersal patterns.

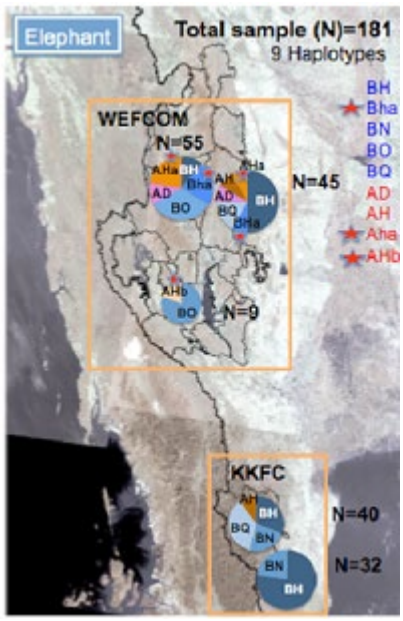

Figure 7. Spatial distribution of 9 mtDNA haplotype across WEFCOM and KKFC. *The Beta clade haplotype included BH, BHa, BN, BO, BQ and Alpha clade included AD, AH, and Aha. The new haplotypes were indicated as red stars. Orange rectangle represented boundary of WEFCOM and KKFC forest complexes separated by farmland and human settlement. The numbers of faecal samples were indicated as N.*

4. Briefly describe the involvement of local communities and how they have benefitted from the project (if relevant).

During our elephant sampling workshops, we gave lectures to local communities, particularly in the high human-elephant conflict zones at the border of Kaeng Krachan National Park. The lecture topics covered: 1) elephant roles as keystone and flagship species, 2) the ancient and current distribution of elephants, 3) current routes of elephant annual and seasonal migration and 4) human land-use change relevant to crop-raiding behavior. Therefore, in addition to engaging local peoples to dialogues of elephant conservation and crop-raiding mitigation, we provided them technical sampling skills and encourage them to become parts of our conservation networks. As stated in 3.1 section of building local capacities, we organized workshops to forestry and veterinary students about the use of integrated genetic and ecological information to inform management decisions.

Figure 8. Local community involvement*: our dedicated networks of conservationists include patrol rangers, forestry students, and young conservationists from village neighboring to protected areas. The picture also showed me giving workshops on sample collection.*

5. Are there any plans to continue this work?

In Thailand and most of Southeast Asia, baseline data about wildlife abundance and population connectivity among habitat fragments remain mostly unknown. The use of crude guess and non-standard methods to estimate abundance have hindered conservation planning, especially human-wildlife conflict mitigation. We strongly believe that integrative landscape genetic, demographic, and ecological data will play an important role in solving conservation issues above.

We will continue our work to understand the underlying evolutionary processes which shape the patterns of spatial genetic diversity in response to rapid environmental changes. We are in the process of completing a detailed analysis of dispersal patterns and inbreeding level of tigers within WEFCOM and of elephants among WEFCOM and KKFC complexes. In addition to tigers and elephants, we will apply both field and laboratory techniques to other threatened mammals including guar (*Bos gaurus*), banteng (*Bos javanicus*), tapir (*Tapirus indicus*), Asiatic black bears (*Ursus thibetanus*) and sun bears (*Ursus manalanus*). Among these species, we already started pilot

sampling of guar and banteng populations across Thailand. We obtained preliminary results of mtDNA diversity. These diversity data will be archived into our national database updated consistently and adjusted to have user-friendly interfaces. Apart from genetic data, the database will have information of each species' demographics, habitats, diseases, all of which would benefit *in situ* and *ex situ* conservation management.

During recent human expansion and domestic animal translocation, wildlife disease surveillance is critical for implementing effective counter measures against infectious and zoonotic diseases. However main problem is early detection of new and emerging disease, stressing the importance of national strategies for wildlife disease detection. Early viral and bacterial detection in fecal samples will tremendously provide insight into the species of disease reservoir and infection dynamics, for example, Avian Influenza viruses in waterbirds.

Advances in sequencing techniques and SNP (Single Nucleotide Polymorphism) analysis allow us to continue developing rapid and efficient genetic screening with inexpensive cost to detect genetic variability. These methods will also facilitate genetic diversity study of ancient specimens from museum collection to make genetic comparison with the current populations. Such comparison will elucidate the effects of human land use change as well as evaluate the efficiency of current conservation management to maintain genetic health of the targeted species. As more data are achieved in our national wildlife genetic database, DNA barcoding system using short and specific DNA sequences as species/subspecies identification will become more accurate. Species identification of illegally imported animal products and assessment of biodiversity of complex communities will be most benefit from this reliable barcoding system.

To develop trans-boundary conservation strategies, we plan to extend sampling areas to neighboring countries to better understand population dispersal dynamics across the remaining range of both tigers and elephants. We will achieve this goal by collaborating with government agencies, NGOs and local students from China and Southeast Asia.

6. How do you plan to share the results of your work with others?

As mentioned in Section 3. I will publish our finding in my PhD thesis at the University of Minnesota in 2014, as well as in the peer-reviewed journal in 2015. In addition, we will provide technical reports, lecture, and workshop to NGO partners (WCS/WWF/FREELAND Foundation), government agencies, and local universities to promote wildlife monitoring based on noninvasive sampling and landscape genetics. This educational programme will strengthen institutional capacity to implement continuous genetic monitoring of the wide-ranging, elusive species whose population dynamics and movement patterns across landscape matrices remain uncertained.

7. Timescale: Over what period was the RSG used? How does this compare to the anticipated or actual length of the project?

Activities	**Plan**	**RSG-funded activities**
Set up a field team and landscape genetic lectures	Mar - Apr 2012	Mar - Jun 2012
Dung collection and preservation	Mar - Sep 2012	Mar - Dec 2012

Genetic analysis: 1) Assess the genetic diversity of tigers and elephants, 2) Identify population genetic structure and estimate gene flow level	Sep 2012 – Feb 2013	Jul 2012 – Jun 2013

8. Budget: Please provide a breakdown of budgeted versus actual expenditure and the reasons for any differences. All figures should be in £ sterling, indicating the local exchange rate used.

Conversion is 48.189 THB/GBP, www.oanda.com

Item	Budgeted Amount	Actual Amount	Difference	Comments
Salary for project leader	202	160	42	The salary was reduced and added to vehicle rental for sample collection as well as to field assistants' food because of extended field work
Stipend for eight field assistants	348	348	0	N/A
Round-trip airfare: Bangkok, Thailand – Beijing, China	250	250	0	N/A
Vehicle rental for sample collection at WEFCOM	508	605	-97	Due to extended field work, funding from project leader salary and genetic laboratory supplies and fee were added to make greater efforts on obtaining fresh fecal samples
Vehicle rental for sample collection at KKFC	156	228	-72	Same reasons as WEFCOM's vehicle rental
Public commute for logistic arrangement	58	58	0	N/A
Field rate per dium for field assistants' food	1015	1215	-200	Extended field work
Dormitory for project leader	225	225	0	N/A
Genetic laboratory supplies and service fee	2912	2600	312	Lower cost due to our newly developed multiplex approach which used less laboratory chemicals and DNA template. Dr. Shujin Luo, Peking University also supported the microsatellite genotyping and mtDNA PCR.
Permit and sample shipment to Peking University, China	44	0	44	Covered by other funding
Handheld GPS Navigator	200	200	0	N/A
Dung collection: Labelling pen	7	7	0	N/A
Dung collection: 50 ml collecting tubes	32	50	-18	To test for efficiency of two preservative methods, one fecal sample was split into two tubes, increasing the required

				tube.
Dung collection: dry desiccant, silica beads	15	26	-11	Same reason as 50 ml collection tubes
Dung collection: Plastic fork	7	7	0	N/A
Dung collection: disposable gloves	21	21	0	N/A
TOTAL	6000	**6000**	0	

9. Looking ahead, what do you feel are the important next steps?

There is a lack of knowledge regarding the remaining genetic diversity, spatial patterns of such diversity, and dispersal patterns across habitat fragments of the wild populations in Asia. We do not know if the current level of gene flow can rescue the populations from genetic threat including genetic drift and inbreeding. To ensure the population viability, understanding how gene flow-drift dynamics influence patterns of genetic variability is urgently needed. We will continue to promote the use of genetic information in national wildlife action plan to achieve effective conservation practices, ensuring the recovery and persistence of the threatened species.

10. Did you use the RSGF logo in any materials produced in relation to this project? Did the RSGF receive any publicity during the course of your work?

Yes, poster presentation at FAVA conference 2013 (Figure. 7).

11. Any other comments?

I am honored to be a recipient of Rufford Small Grant. Your generous support helps me and my teams fulfill our goal of implementing landscape genetic monitoring in Thailand's conservation strategies. This grant is critical for me to gain multidisciplinary research experiences as well as contribute back to the graduate community and general public via outreach engagement and academic talk. My long-term goal is to identify how interactions between evolutionary processes and landscape mosaics shape genetic variability and dispersal dynamics. In the face of rapid environmental changes, a better understanding of these interactions will lead to effective management of endangered species. My research project supported by your fellowship will help the recovery of not only tigers and elephants, but the species urgently needed for protection.