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	Milena Salgado Lynn
Project title	Conservation of adaptive genetic diversity in primates of the Lower Kinabatangan Wildlife Sanctuary
RSG reference	34.01.07
Reporting period	October 2007-November 2008
Amount of grant	£2,398.00
Your email address	Salgado-Lynn Milena
Date of this report	January 2009

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
Obtention of DNA Samples				1st year objective. Successfully achieved. Enriched by the training of staff of Red Ape Encounters (community based Ecotours) and joint work with a Wildlife Warden from the Sabah Wildlife Department who reported all the illegal activities witnessed during the sampling season.
• Non-invasive sampling			x	A total of 1161 faecal samples were collected throughout the Lots of the Lower Kinabatangan Wildlife Sanctuary (in total, the surveys covered ~600 km of riverbank including smaller tributaries and oxbow lakes).
• DNA Extractions			x	A total of 558 DNA extracts were obtained.
Measurement of parasite loads and prevalence				1st year objective. Partially achieved. A collaboration with Prof. Michael Muehlenbein from Indiana University was established for the morphological identification of parasites. Red Ape Encounters staff was trained in parasite isolation and identification techniques. The samples from the first season (Lots 1-4) were successfully processed, while work on the samples from the second season (Lots 5-10c) is still ongoing.
• Isolation & identification of parasitic fauna		x		Two techniques were tested to isolate parasite eggs. Clear identification of 4 helminthic types was achieved and identification of parasitic-like objects is currently ongoing.
• Estimation of parasitic loads		x		Awaiting confirmation of parasite identity to continue statistical analysis.
• Estimation of prevalence		x		Awaiting confirmation of parasite identity to continue statistical analysis.
Genotyping neutral molecular markers (for proboscis monkeys and long tailed macaques)				2nd year objective. The second year of the project is initiating. Work on the objectives for this part of the project is ongoing.
• Cross amplification with human			x	Unsuccessful cross-amplification of human microsatellite primers in proboscis monkey. A new approach

microsatellite primers				has been devised and work in a microsatellite library to develop species-specific primers is being carried out. Cross-amplification in long-tailed macaque samples has not yet been attempted.
• Determination of neutral genetic variation			x	
Genotyping adaptive molecular markers				2nd year objective. Programmed for the second half of this year.
• Development of MHC markers (for proboscis monkeys, long tailed macaques and orang-utans).			x	
• Determination of adaptive genetic variation			x	
Comparison of levels of microsatellite and MHC variations			x	3rd year objective.
Determination of relatedness between parasite loads and prevalence with levels of MHC Variation			x	3rd year objective.
Incorporation of genetic results into Conservation Management Plan			x	3rd year objective.

Summary of the project:

The Lower Kinabatangan Wildlife Sanctuary (LKWS) in Sabah, Malaysia, provides a broad diversity of habitats allowing 10 primate species to live in sympatry. The area is considered of conservation priority, unfortunately, the landscape has been drastically altered by human activities. The habitat is highly fragmented, and the LKWS is now composed of isolated patches of forests (Lots) with different physical and biological attributes. This habitat loss and fragmentation could pose extinction threats to primate populations by the loss of genetic diversity and the emergence of infectious diseases. Consequently monitoring the populations' genetic diversity and health is more than pertinent.

The study of endangered species requires approaches that minimize the handling of animals for acquisition of samples. Molecular scatology is ideal for studying endangered species since invasive sampling is prohibited by law. Moreover, the use of faecal samples has proved successful in a range of species, including primates such as gorillas, chimpanzees, baboons and langurs (Bayes et al., 2000; Bradley et al., 2000; Launhardt et al., 1998; Little, 2003; Morin et al., 2001). As a matter of fact, all the previous primate genetic studies in the LKWS have been successfully achieved through non-invasive collection of samples (Goossens et al., 2005; 2006). In particular, the mtDNA studies on proboscis monkeys and macaques were performed using DNA extracted from faecal samples (Jalil, 2007), and this information is used as a basis for the current project.

The advent of PCR has lead to the development of several diagnostic assays that target the various stages of parasitic nematodes (Harmon et al., 2007). However, to meet the needs of such assays,

fixation should allow for: preservation of egg morphology, normal egg recovery through faecal flotation and uninhibited PCR with egg-derived DNA. Flotation techniques that rely on the specific gravity differences between parasite eggs and faecal debris are the most frequently used methods to recover parasites from stool samples (Dryden et al., 2005). These techniques are useful if the eggs are required for culturing as it has little effect on their viability (FAO, 2007), or as a means of preserving DNA, in contrast to more generalised fixation techniques that rely on formaldehyde, which preserve egg morphology longer but inhibit PCR reactions (Harmon et al., 2007). The gastrointestinal parasites of Kinabatangan primates are unknown therefore morphological characterisation is an essential prerequisite for molecular studies. However, preservation of DNA is important in case species-specific primers need to be designed for such studies.

The use of microsatellite markers to study population genetic structure has been widely applied. An advantage of this type of markers is the possibility of using primers developed in one species to amplify homologous loci between related taxa (Bruford & Wayne, 1993; Jarne & Lagoda, 1996). Human microsatellite primers have successfully been used in other non-human primates, including all the great apes and several old-world monkeys (Bayes et al., 2000, Bonhomme et al., 2005; Coote & Bruford, 1996; Goossens et al., 2000; Launhardt et al., 1998; Little, 2003; Roeder et al., unpublished results).

Genes of the Major Histocompatibility Complex (MHC) are thought to be of important adaptive significance and have thus been used as indicators of adaptive genetic variability (Robinson et al., 2003). Particularly, MHC-DRB gene exon 2 is of special interest because it codes for the antigen recognition and binding sites (ABS) (Hughes & Yeager, 1998). However, determination of MHC haplotypes and homozygosity can be problematic even when using “invasive” samples (Knapp, 2005). For that reason, it has been suggested to apply considerations similar to those used when typing microsatellites from non-invasive samples (Goossens et al,. 1998; Knapp, 2005 Taberlet et al., 1996).

This project will complement existing data by assessing the level of adaptive genetic variability within and between sub-populations of long-tailed macaques, orang-utans and proboscis monkeys. The variability of the Major Histocompatibility Complex Class II (MHC II) genes will be evaluated, focusing on the DRB exon 2 locus. This is the first genetic study in the LKWS to address selective environmental pressures upon the three species. The level of MHC variation will then be correlated with natural selection via parasite (nematode) diversity and load. Moreover, the genetic datasets for proboscis monkeys and long-tailed macaques will be supplemented by genotyping microsatellites, which in proboscis monkeys has not been previously attempted. A comparison between the neutral (microsatellites) and the adaptive (MHC) genetic variation will also be carried out. The information will be used to identify genetic differentiation between sub-populations and threats to genetic diversity in order to determine appropriate intervention measures to maintain gene flow and diversity between these sub-populations. The results will be incorporated into the conservation management plan of these primates to ensure their long term-survival.

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

The Rufford Small Grant was awarded to carry out a different project (Simian Malaria in the Lower Kinabatangan Region in Sabah, Malaysia) which had to be cancelled due to unexpected circumstances. The Rufford Small Grants Foundation was notified and an agreement to pursue the current project was achieved. Although aware of the lack of parasite data of Kinabatangan primates, the difficulty of identifying specimens surpassed our capacities. Prof. Michael Muehlenbein, from Indiana University, has been collaborating with the Kinabatangan Orangutan Conservation Project (KOCP) and the Sabah Wildlife Department (SWD) regarding a programme on ecosystem health monitoring. He kindly agreed to collaborate in this project by confirming our findings and/or identifying specimens.

Regarding the genetic work, the inability to amplify microsatellites in DNA from proboscis monkeys using human primers has set up the challenge of developing species specific primers. We are constructing a microsatellite library for proboscis monkey, which is the first of its kind.

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

This project was devised to run over three years, at the end of the first year these are the most important outcomes:

- The involvement of the local community through the support of Red Ape Encounters was the most important outcome. Training the staff, particularly Mr. Rosman Sakong, to collect samples and carry out the parasite isolation and identification is important for the ecosystem health monitoring project that is about to start, and which will last at least five years.
- There were no records for parasitic fauna in proboscis monkeys overall, or for Kinabatangan primates. These will be the first reports regarding this subject, which will set a basis for future monitoring on health status of the primate populations.
- During the sampling throughout the sanctuary, we witnessed anomalies, which were reported to the Sabah Wildlife Department by an Honorary Wildlife Warden, Dr. Benoît Goossens (Director of Danau Girang Field Centre). In this sense, we notified of bird smugglers, small camps in the protected forest, waste dumps in the shore of the Kinabatangan River, and more strikingly the lack of proboscis monkey groups in a big area of the Sanctuary. The Sabah Wildlife Department was keen on setting up an investigation, which is currently being pursued.

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

See above.

5. Are there any plans to continue this work?

As mentioned, this is a three year project and the second year has just started.

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

All outcomes will be published in peer reviewed journals, and data will also be presented at scientific meetings to be critically assessed by our peers.

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

The Rufford Small Grant was used to carry out 10 months of fieldwork in Sabah (Oct 2007-Feb 2008, Jun – Oct 2008). This stage is now concluded and constitutes one third of the actual length of the project. The original budget modified drastically, since we applied for a different project with very different costs. We had planned only one sampling season of 6 months, but as the project changed

this had to be extended to two seasons of five months each. For that reason, travel expenses, equipment and consumables also changed.

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.

From Oct 2007 to Nov 2008, Malaysian Ringgit fluctuated between RM6.7 and RM5.3 to the pound, therefore we used RM6.00 as mean exchange rate.

Item	Budgeted Amount	Actual Amount	Difference	Comments
Transportation	£1263.00	£831.85 (spent from the RSG)	£431.15	We were awarded a British Society for Parasitology Garnham Travelling Award, which was used to cover this item. The remainder was used to cover consumables.
Salaries	£500.00	£417.67	£82.33	Occasionally during the first sampling season, and throughout the second sampling season we were aided by volunteers, which reduced the cost of salaries.
Equipment	£635.00	£249.95	£385.05	The initial budget was to cover mosquito traps and camping gear, among others. As the project changed such equipment was not needed, and through the collaboration with Danau Girang Field Centre camping gear was procured.
Consumables	£0.00	£908.88	£908.88	The new project involved collection of faecal material, which needs a very different type of storage than mosquitoes. In that sense, tubes, ethanol, and big storage boxes had to be bought. Additionally, DNA needed to be extracted in situ to avoid customs restrictions, so a whole set of laboratory consumables (tips, tubes) and extraction kits were also acquired. QIAGEN DNA stool extraction kits cost £105.00 for 50 reactions.
TOTAL	£2398.00	**£2409.35**	£11.35	

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

Conclude the genetic area of the project and the statistical analysis of the parasite data is the most important step now. Those results will allow us to make correlation and predictions that could be used by the Sabah Wildlife Department for the conservation management plan of the species.

Simultaneously, it is important to keep the collaboration with Red Ape Encounters, so the local communities are aware of the status of the wildlife in order to help to their conservation.

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. A poster was presented on the outline of the project at the Biodiversity and Ecological Processes Group Symposium (2007), and at the School of Biosciences Poster Evening (2008) of Cardiff University. In addition, partial results were presented orally at the Biodiversity and Ecological Processes Group Symposium (2008).

11. Any other comments?

At this stage of the project, there are still no concluding results. However, we have mostly achieved the goals set for the first year of the project. On due course, relevant results will arise and a final report on the whole project will be presented to The Rufford Small Grants Foundation.

References

Bayes MK, Smith KL, Alberts SC, Altmann J, Bruford MW. 2000. Testing the reliability of microsatellite typing from faecal DNA in the savannah baboon. Conservation Genetics, 1: 173-76.

Bonhomme M, Blancher A, Crouau-Roy B. 2005. Multiplexed microsatellites for rapid identification and characterization of individuals and populations of Cercopithecidae. American Journal of Primatology, 67: 385-91.

Bradley BJ, Boesch C, Vigilant L. 2000. Identification and redesign of human microsatellite markers for genotyping wild chimpanzee (Pan troglodytes verus) and gorilla (Gorilla gorilla gorilla) DNA from faeces. Conservation Genetics, 1: 289-92.

Bruford MW and Wayne RK. 1993. Microsatellites and their application to population genetic studies. Current Opinions in Genetics and Development, 3: 939-43.

Coote T and Bruford MW. 1996. Human microsatellites applicable for analysis of genetic variation in apes and old world monkeys. The Journal of Heredity, 87: 406-10.

Dryden MW, Payne PA, Ridley R, and Smith V. 2005. Comparison of common fecal flotation techniques for the recovery of parasite eggs and oocysts. Veterinary therapeutics, 6: 15-28.

Food and Agriculture Organization (FAO) of the United Nations. 2007. The RVC/FAO guide to veterinary diagnostic parasitology: Faecal examination of farm animals for helminth parasites. Compiled by: Gibbons LM, Jacobs DE, Fox MT and Hansen J. Retrieved 5 July 2007, from: http://www.rvc.ac.uk/review/Parasitology/Index/Index.htm

Goossens B, Chikhi L, Jalil MF, Ancrenaz M, Lackman-Ancrenaz I, Mohamed M, Andau P, Bruford W. 2005. Patterns of genetic diversity and migration in increasingly fragmented and declining orang-utan (Pongo pygmaeus) populations from Sabah, Malaysia. Molecular Ecology, 14: 441-56.

Goossens B, Setchell JM, James SS, Funk SM, Chikhi L, Abulani A, Ancrenaz M, Lackman-Ancrenaz I, Bruford MW. 2006. Philopatry and reproductive success in Bornean orang-utans (Pongo pygmaeus). Molecular Ecology, 15: 2577-88.

Goossens, B, Chikhi L, Utami S, de Ruiter J, Bruford MW. 2000. A multi-samples, multi-extracts approach for microsatellite analysis of faecal samples in an arboreal ape. Conservation genetics, 1: 157-62.

Harmon AF, Williams ZB, Holler LD, Hildreth MB. 2007. Comparison of three different preservatives for morphological and real-time PCR analyses of Haemonchus contortus eggs. Veterinary Parasitology, 145: 361-365.

Hughes AL, Yeager M. 1998. Natural selection at major histocompatibility complex loci of vertebrates. Annual Review of Genetics, 32: 415-35.

Jalil MFB. 2007. Comparative phylogeography of three primate species in the Lower Kinabatangan Wildlife Sanctuary, Sabah, Malaysia. Thesis submitted for the degree of Doctor of Philosophy. School of Biosciences, Cardiff University. pp. 201.

Jarne P and Lagoda PJL. 1996. Microsatellites, from molecules to populations and back. Trends in Ecology and Evolution, 11: 424-29.

Knapp LA. 2005. Facts, faeces and setting standards for the study of MHC genes using noninvasive samples. Molecular Ecology, 14: 1597-99.

Launhardt K, Epplen C, Epplen JT, Winkler P. 1998. Amplification of microsatellites adapted from human systems in faecal DNA of wild Hanuman langurs (Presbytis entellus). Electrophoresis, 19: 1356-61.

Little KA. 2003. Genetic relatedness and socioecology of wild Hanuman langur monkeys. PhD Thesis, University College London, University of London.

Morin PA, Chambers KE, Boesch C, Vigilant L. 2001. Quantitative polymerase chain reaction analysis of DNA from non-invasive samples for accurate microsatellite genotyping of wild chimpanzees (Pan troglodytes verus). Molecular Ecology, 10: 1835-44.

Robinson J, Waller MJ, Parham P, de Groot N, Bontrop R, Kennedy LJ, Stoehr P, Marsh SGE. 2003. IMGT/HLA and IMGT/MHC: sequence databases for the study of the major histocompatibility complex. Nucleic Acids Research, 31: 311-14.

Taberlet P, Griffin S, Goossens B, Questiau S, Manceau V, Escaravage N, Waits LP, Bouvet J. 1996. Reliable genotyping of samples with very low DNA quantities using PCR. Nucleic Acids Research, 24: 3189-94.