CEPF Final Project Completion Report

Instructions to grantees: please complete all fields, and respond to all questions, below.

Organization Legal Name	Centre for Natural Resources and Environmental Studies
Project Title	Strengthening conservation of the most critically endangered turtles in Vietnam through application of environmental DNA
CEPF GEM No.	029
Date of Report	November 14, 2016

CEPF Hotspot: Indo-Burma

Strategic Direction: CEPF Strategic Direction 1.1 and 1.2

Grant Amount: 19,990

Project Dates: October 2014 to October 2016

1. Implementation Partners for this Project *(list each partner and explain how they were involved in the project)*

Cleveland Metropark Zoo's Asian Turtle Program (ATP): Scientific staff from ATP actively participated in all surveys from planning to conducting interviews and fieldwork. In total, CRES and ATP carried out one interview survey in Quang Ninh Province, two eDNA sample collecting trips to Quang Nam and Quang Ngai Provinces.

<u>Conservation Impacts</u>

2. Describe how your project has contributed to the implementation of the CEPF investment strategy set out in the ecosystem profile

The project has helped to survey and improve information on distribution and abundance of the Zhou's Box Turtle (*Cuora zhoui*) and the Vietnam's Pond Turtle (*Mauremys annamensis*), two CEPF priority species, in their potential distribution sites.

3. Summarize the overall results/impact of your project

The project has enhanced scientific knowledge of the targeted turtle species. Collaboration between related stakeholders working on conservation has been strengthened during the process of the project implementation. The research team has gained valuable experience in surveying the critically endangered species, especially applying eDNA techniques to studying elusive species in aquatic habitat. Specifically, we successfully tested the techniques in laboratory facilities for the Four-eyed Turtle (*Sacalia quadriocellata*), and were able to confirm the presence of the Oldham's Leaf Turtle (*Cyclemys oldhamii*) in the field. These skills are essential in developing conservation activities for critically endangered species in Vietnam.

During the course of the project, we undertook an interview survey in Quang Ninh Province. In total, 120 interviews were conducted with 15 species being identified in the survey area. Notably, the critically endangered Swinhoe's Softshell Turtle (*Rafetus swinhoei*) was reported for the first time in the province. The information will need to be confirmed by more in-depth surveys in the area. In addition, the Malayan Snail-eating Turtle (*Malayemys subtrijuga*), which originates from southern Vietnam, was located in a trader house in Hoanh Bo District. This suggests that the turtle trade network is operating very effectively in Vietnam.

During our interview and field surveys, we are able to identify the presence of a number of high conservation priority species in Quang Ninh Province, e.g., the Big-headed Turtle (*Platysternon megacephalum*), the Indochinese Box Turtle (*Cuora galbinifrons*), the Keeled Box Turtle (*Cuora mouhotii*), the Chinese Three-striped Box Turtle (*Cuora trifasciata*), and Yellow Pond Turtle (*Mauremys mutica*), the Chinese Stripe-necked Turtle (*Mauremys sinensis*), the Wattle-necked Softshell Turtle (*Palea steindachneri*), and the Four-eyed Turtle (*Sacalia quadriocellata*). The data can help prioritize conservation efforts in the future for the threatened chelonian species.

Planned Goal (as stated in the approved proposal)

Wild populations of Zhou's Box Turtle and the Vietnam Pond Turtle are safeguarded in Vietnam, based on improved information about their distribution and abundance.

4. Actual progress toward Goal at completion

We were unable to locate any population of the Zhou's Box Turtle in the wild even though we conducted in total 253 interviews, including those collected by previous projects, in three provinces, Bac Kan, Quang Ninh, and Tuyen Quang Provinces, and processed 31 water samples from Bac Kan Province.

Similarly, no wild population of the Vietnam's Pond Turtle was identified, although we processed 149 water samples from all potential sites of the species in Phu Yen, Quang Nam, and Quang Ngai Provinces.

Planned Objectives (as stated in the approved proposal)

1. The location of populations of Zhou's Box Turtle in Vietnam is understood

2. The location of populations of the Vietnam Pond Turtle is understood

5. Actual progress toward Objectives at completion

In total, 180 water samples from all potential sites of the Zhou's Box Turtle and the Vietnam's Pond Turtle were processed. No DNA trace of the two species was found in the sites. We provisionally conclude that the sites do not harbor any populations of the two species.

6. Describe the success or challenges of the project toward achieving its goal and objectives

It is an extremely challenging task to determine wild populations of the two critically endangered species. The Zhou's Box Turtle has never occurred in high abundance, and since its description, no information on its distribution has been reported. Moreover, with immense

pressure from hunting and its high economic value, it is even more difficult to locate the species in the wild.

The Vietnam's Pond Turtle used to occur in higher abundance. Nonetheless, sustaining hunting pressure on the species endemic to a small lowland region in central Vietnam has extirpated many local populations of the species. In recent years, the species has reached a disproportionately high price, making it a special target for local people. Surveys of the species are therefore very challenging.

7. Were there any unexpected impacts (positive or negative)?

No

Project Activities and Deliverables

Objective 1 (as stated in the approved proposal)

The location of populations of Zhou's Box Turtle in Vietnam is understood

Objective 2 (as stated in the approved proposal)

The location of populations of the Vietnam Pond Turtle is understood

8. Describe the activities implemented and deliverables met under Objective 1

Activity 1.1. Collect environmental DNA (eDNA) samples from sites potentially containing populations of the Zhou's Box Turtle

We collected samples from all potential sites in Bac Kan Province.

Activity 1.2. Analyze eDNA samples for presence of the Zhou's Box Turtle DNA

Our interview surveys yielded very limited information on the species. As a result, we were unable to collect more water samples for DNA analyses. In total, we analyzed 31 water samples collected from Bac Kan Province. We did not find DNA of the Zhou's Box Turtle, but found DNA of the Oldham's Leaf Turtle in one of the sites.

9. Repeat point 8 above for each Objective in your approved proposal

Activity 1.1. Collect eDNA samples from sites potentially containing populations of the Vietnam Pond Turtle

We collected samples from all potential sites in Phu Yen, Quang Nam, and Quang Ngai Provinces.

Activity 1.2. Analyze eDNA samples for presence of Vietnam Pond Turtle DNA

In total, we analyzed 149 water samples collected in three provinces. No DNA of the Vietnam's Pond Turtle was identified.

10. If you did not complete any activity or deliverable, how did this affect the overall impact of the project?

11. Please describe and submit any tools, products, or methodologies that resulted from this project or contributed to the results

In the first phase of the project, we collected two 300ml water samples from a plastic water tank (610 × 420 × 190 mm) where a small individual (0.4 kg) of the Four-eyed Turtle (*Sacalia quadriocellata*) was kept for 2 days in about 20L of water. Water samples were filtered with a pump (Silverline elite automotive tetst kit model MV8500) through 0.45 µm cellulose nitrate filters (disposable filter funnel, 47 mm grindded filter, Whatman product no. 7184-004). Each filter was preserved in 70% ethanol in a sterile 2 ml tube then stored at -20^{o}C until DNA extraction.

DNA was extracted from the filtered samples using the Dneasy Blood and Tissue Kit (Quiagen, Germany) following the manufacturer's protocol. For the first step, we removed the filters from the ethanol and air-dried them overnight. For the incubation step, the lysis usually took up to 48h for the filter samples to be dissolved completely. After the step, we centrifuged the samples to spin down the solutions. Final elutes were stored in 40µl Elution Buffer. One half of the filter was used for each extraction. The remaining half was stored in the freezer. DNA extraction was carried out in a clean room using a BioHazard Safety Cabinet (Daihan Labtech Batam, Indonesia). We then amplified DNA from two samples using a standard PCR protocol with HotStarTaq Mastermix (Quiagen). The standard PCR conditions were amplified under the condition: 95^{o}C for 15min to active Taq, 35 cycles at 95^{o}C for 30s, 45^{o}C for 45s, 72^{o}C for 60s; a final elongation at 72^{o}C for 6min. The PCR components were: 2µl of each primer at 10 µmol/µl, 5µl water, 10µl of HotStarTaq Mastermix and 2µl DNA template. The primers used to amplify a fragment of the mitochondrial DNA (mtDNA) *12S* gene using a pair of primers L1091 (5'-AAACTGGGATTAGATACCCCACTAT-3') and H1478 (5'-GAGGGTGACGGGCGGTGTGT- 3') (Kocher et al. 1989). To confirm PCR products, 5µl of each sample was run on a 1% agarose gel, 1X TBE buffer, stained with 2pg/µl Ethidium bromide and photographed under UV light. PCR products were cleaned using Gene Jet PCR Purification Kit (Thermo Fisher Scientific) following the manufacturer's instructions. The cleaned PCR products were sent to 1st Base (Malaysia) for sequencing.

In the second phase of the project, 400 – 500ml water samples were collected in 12 natural ponds/lakes where *Mauremys annamensis* and *Cuora zhoui* were expected to present based on interview surveys (Table 1). For each sample site, we collected 4 – 8 samples from different sites depending on size of wetlands and numbers of potential sites. Water samples were vacuum – filtered within 72h of collection through 0.45 µm cellulose nitrate filters (disposable filter funnel, 47 mm grindded filter, Whatman product no. 7184-004). Each filter was preserved in 70% ethanol in a sterile 2 ml tube then stored at -20^{o}C until DNA extraction. We used the above extraction method for all of the samples, but modified the final step. We diluted trapped DNA in

30µl Elution Buffer, because the DNA concentration was much lower than the that in samples collected from the water tank in the laboratory.

We designed 9 specific primers for turtles targeting a small region of the mitochondrial DNA (mtDNA) NADH dehydrogenase subunit 4 (*ND4*) gene. After testing all the primers, the following primer pair, TeM1 (5' - GCCAAACAGACCTAAAATCATTA - 3') and TeM2 (5' – GGCAGAAAAGTATTGATGATGTT - 3'), was selected. Quantitative polymarase chain reaction (qPCR) amplification was employed to amplify a 150 bp fragment of mitochondrial ND4 gene using qPCRBIO SyGreen Mix Lo-Rox (Labtech). The qPCR volume consisted of 16µl (0.8µl each primer, 4.2µl water, 10µl of qPCRBIO SyGreen Mix, 3µl DNA template). PCR conditions were 95^{o}C for 3min to active Mastermix, 55 cycles at 95^{o}C for 15s and 60^{o}C for 30s, 1 cycles of melting curve at 95^{o}C for 15s. The samples collected from the water tank and the other samples of *Mauremys annamensis* collected in Quang Nam Province were used as positive control. A negative control was used in every qPCR reaction. Samples were run on PCR tube strips (ThermoFisher Scientific) on an Applied Biosystems StepOne^{TM} Real – Time PCR Systems (ThermoFisher Scientific). Analysis of qPCR data was conducted using StepOne^{TM} Software v2.2.2. The qPCR products were considered negative if no exponential phase occurred during 55 cycles. Positive qPCR products were sent to 1st Base (Malaysia) for purifying and sequencing.

Table 1. Localities of samples used in this study

Location (Province)	Number of sites	Number of samples	ID	Expected species
Bac Kan	4 (An Tinh, Quang Phong, Cho Moi, Duong Son Commune)	31	EC1 - EC31	*C. zhoui*
Phu Yen	1 (Song Hinh District)	7	Ms1 - Ms7	*M. annamensis*
Quang Nam	3 (Dien Ban District)	30	Ms80 – Ms109	*M. annamensis*
Quang Ngai	7 (Binh Son Commune)	112	Ms8 – Ms79 Ms110-Ms149	*M. annamensis*

Benefits to Communities

12. Please describe the communities that have benefited from CEPF support

*Please report on the size and characteristics of communities and the benefits that they have received, as a result of CEPF investment. Please provide information for all communities that have benefited **from project start to project completion**.*

	Community Characteristics											Nature of Socioeconomic Benefit								
								Size of Community												
Community Name	**Subsistence economy**	**Small landowners**	**Indigenous/ ethnic peoples**	**Pastoralists / nomadic peoples**	**Recent migrants**	**Urban communities**	**Other***	**50-250 people**	**251-500 people**	**501-1,000 people**	**Over 1,001 people**	**Increased access to clean water**	**Increased food security**	**Increased access to energy**	**Increased access to public services (e.g. health care, education)**	**Increased resilience to climate change**	**Improved land tenure**	**Improved recognition of traditional knowledge**	**Improved representation and decision-making in governance forums/structures**	**Improved access to ecosystem services**
	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No	No

*If you marked "Other" to describe the community characteristic, please explain:

Lessons Learned

13. Describe any lessons learned related to organizational development and capacity building.

Through the implementation of the project, CRES has been able to build capacity in applying eDNA techniques to surveying aquatic species. In addition to more conventional PCR techniques, we have been able to master the qPCR or real-time PCR technology, which is more sensitive than the conventional one.

14. Describe any lessons learned related to project Design Process *(aspects of the project design that contributed to its success/shortcomings)*

No

15. Describe any lesson learned related to project Implementation *(aspects of the project execution that contributed to its success/shortcomings)*

The project was delayed for several months because we wanted to try the new real-time PCR technique, which is more effective than conventional PCR. Employing real-time PCR helped us to identify DNA of the Oldham's Leaf Turtle in Bac Kan Province. This early success shows that this survey technique can be a great tool in supporting surveys of elusive species in aquatic environment.

16. Describe any other lessons learned relevant to the conservation community

It is likely that the Zhou's Box Turtle and the Vietnam's Pond Turtle have gone extinct in the wild, because repeated survey efforts were unable to locate any populations of the species. Future *in-situ* conservation programs of the species will need to rely on reintroduction of captive colonies. Before reintroduction can be undertaken, protected areas for the species should be established to ensure that they are protected under an appropriate legal framework.

Sustainability / Replication

17. Summarize the success or challenges in ensuring the project will be sustained or replicated

The eDNA techniques developed through this project can be used in future survey efforts to identify wild populations of elusive and endangered species in aquatic habitat. In addition, the project helped to train a young master's student in application of the techniques. The outcomes of the project will be sustained and replicated in future conservation studies of these or other aquatic species in the region.

18. **Summarize any unplanned activities that are likely to result in increased sustainability or replicability**

No

Safeguards

19. If not listed as a separate Project Component and described above, summarize the implementation of any required action related to social and environmental safeguards that your project may have triggered

No

Additional Funding

20. Provide details of any additional funding that supported this project and any funding secured for the project, organization, or the region, as a result of CEPF investment

Donor	Type of Funding*	Amount	Notes
Rotterdam Zoo	A	$2,800	

** Categorize the type of funding as:*

A Project Co-Financing (other donors or your organization contribute to the direct costs of this project)
B Grantee and Partner Leveraging (other donors contribute to your organization or a partner organization as a direct result of successes with this CEPF funded project)
C Regional/Portfolio Leveraging (other donors make large investments in a region because of CEPF investment or successes related to this project)

Additional Comments/Recommendations

21. Use this space to provide any further comments or recommendations in relation to your project or CEPF

No

Information Sharing and CEPF Policy

CEPF is committed to transparent operations and to helping civil society groups share experiences, lessons learned, and results. Final project completion reports are made available on our Web site, www.cepf.net, and publicized in our newsletter and other communications.

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