Conservation Biology

Contributed Paper

Conservation genomics of the endangered Burmese roofed turtle

F. Gözde Çilingir,[1] Frank E. Rheindt,[1]* Kritika M. Garg,[1] Kalyar Platt,[2] Steven G. Platt,[3] and David P. Bickford[4]

[1]Department of Biological Sciences, National University of Singapore, 16 Science Drive 4 117558, Singapore
[2]Turtle Survival Alliance, Myanmar Program, Office Block C-1, Aye Yeik Mon 1st Street, Hlaing Township, Yangon, Myanmar
[3]Wildlife Conservation Society, Office Block C-1, Aye Yeik Mon 1st Street, Hlaing Township, Yangon, Myanmar
[4]Department of Biology, University of La Verne, 1950 Third Street, La Verne, CA 91750, U.S.A.

Abstract: *The Burmese roofed turtle* (Batagur trivittata) *is one of the world's most endangered turtles. Only one wild population remains in Myanmar. There are thought to be 12 breeding turtles in the wild. Conservation efforts for the species have raised >700 captive turtles since 2002, predominantly from eggs collected in the wild. We collected tissue samples from 445 individuals (approximately 40% of the turtles' remaining global population), applied double-digest restriction-site associated DNA sequencing (ddRAD-Seq), and obtained approximately 1500 unlinked genome-wide single nucleotide polymorphisms. Individuals fell into 5 distinct genetic clusters, 4 of which represented full-sib families. We inferred a low effective population size (≤10 individuals) but did not detect signs of severe inbreeding, possibly because the population bottleneck occurred recently. Two groups of 30 individuals from the captive pool that were the most genetically diverse were reintroduced to the wild, leading to an increase in the number of fertile eggs (n = 27) in the wild. Another 25 individuals, selected based on the same criteria, were transferred to the Singapore Zoo as an assurance colony. Our study demonstrates that the research-to-application gap in conservation can be bridged through application of cutting-edge genomic methods.*

Keywords: *Batagur trivittata*, Burma, conservation genomics, ddRAD-Seq, Myanmar

Genética de la Conservación de la Tortuga Rugosa Birmana en Peligro de Extinción

Resumen: *La tortuga rugosa birmana* (Batagur trivittata) *es una de las tortugas en mayor peligro de extinción de todo el mundo. En Myanmar sólo permanece una población silvestre. Se cree que existen 12 tortugas reproductoras en vida libre. Los esfuerzos de conservación para la especie han criado >700 tortugas en cautiverio desde 2002, principalmente a partir de huevos recolectados del hábitat natural. Colectamos muestras de tejido de 445 individuos (aproximadamente 40% de la permaneciente población mundial de la tortuga), aplicamos una secuenciación de ADN asociado de digestión-doble restringida al sitio (ddRAD-Seq, en inglés) y obtuvimos aproximadamente 1500 polimorfismos de nucleótido único, sin conexión y del ancho del genoma. Los individuos cayeron dentro de cinco agrupamientos genéticos distintos, cuatro de los cuales representaron familias de hermanos completos. Inferimos un tamaño reducido de población efectiva (≤10 individuos) pero no detectamos señales de endogamia severa, posiblemente porque el cuello de botella poblacional ocurrió recientemente. Dos grupos de 30 individuos del pool en cautiverio, que fueron los más diversos genéticamente, fueron reintroducidos a su hábitat natural, resultando en un incremento del número de huevos fértiles (n = 27) en vida libre. Otros 25 individuos, seleccionados con base en los mismos criterios, fueron transferidos al Zoológico de Singapur como una colonia de garantía. Nuestro estudio demuestra que el vacio de investigación-a-aplicación en la conservación puede remediarse por medio de la aplicación de métodos genómicos innovadores.*

Palabras Clave: *Batagur trivittata*, Birmania, ddRAD-Seq, genética de la conservación, Myanmar

*email dbsrfe@nus.edu.sg
Paper submitted October 14, 2016; revised manuscript accepted February 17, 2017.

Conservation Biology, Volume 31, No. 6, 1469–1476

DOI: 10.1111/cobi.12921

Introduction

Turtles are among the vertebrate groups most affected by the Anthropocene defaunation (Hoffmann et al. 2010; Dirzo et al. 2014). Asian turtles in particular are seriously threatened given that almost 70% of the world's 25 most critically endangered turtles occur in Asia (Turtle Conservation Coalition 2011).

The Burmese roofed turtle (*Batagur trivittata*) is endemic to the larger rivers of Myanmar (Fig. 1 & Supporting Information). Presumed extinct by the late 1990s, *B. trivittata* was rediscovered in 2001 and is now considered among the 5 most endangered chelonians (Platt et al. 2005; Kuchling et al. 2006*a*). Two remnant wild populations were identified in the early-2000s, one in the Chindwin River Basin (upper Chindwin River [Kuchling et al. 2006*b*]) and the other in the Dokthawady River System (Kuchling et al. 2006*a*). However, a subsequent survey in 2011 upstream from a newly constructed hydroelectric dam and impoundment showed that the only sandbanks where *B. trivittata* was known to nest had been inundated (Platt & Soe 2011); thus, wild individuals of the species were restricted to a population of <10 females (based on annual nesting effort) inhabiting a remote stretch of the upper Chindwin River in western Myanmar (Turtle Conservation Coalition 2011) (Fig. 1).

Ex situ conservation of *B. trivittata* was initiated with 2 females of unknown origin rescued in 2002 as subadults from the Mahamuni Paya pond in Mandalay (Kuchling & Lwin 2004). In addition to these individuals, the current assurance colony includes an adult male and female, confiscated from a trader at the Chindwin River, and 2 males and 1 female from the Dokhtawady River population obtained from fishers (Kuchling et al. 2006*a*; Platt et al. 2013).

Since 2006, recovery efforts have integrated in situ conservation measures, including the protection of turtles and remaining nesting sites in the upper Chindwin River, with ex situ measures such as the transfer of clutches to secure incubation sites, head starting of hatchlings, and captive breeding of founders assembled in 2002–2004. The objective of these measures has been to restore a viable population of *B. trivittata* along the Chindwin River. As a result, the global population of *B. trivittata* currently numbers >700 (most in captivity), and the species seems reasonably secure from biological extinction. Despite conservation progress, virtually nothing is known of the ecology, behavior, and genetic diversity or structure of *B. trivittata*. Such information is critical for developing effective, scientifically informed conservation and management plans (Dayton 2003), and reintroduction is the next phase of this ongoing conservation effort.

Using genome-wide single nucleotide polymorphisms (SNPs) obtained from double-digest-restriction-associated DNA sequencing (Peterson et al. 2012) (ddRAD-seq), we sampled approximately 40% of the remaining global population of *B. trivittata* to determine genetic structure and measure genetic diversity; to conduct parentage analysis to estimate numbers of males and females contributing to the current gene pool and to measure effective population size; and to inform genetic management of reintroduction and assurance colonies (i.e., increase genetic diversity of the remaining wild population). We also sought to provide an illustration of how to bridge the rift between academic conservation science and conservation action (Shafer et al. 2015; Corlett 2017).

Methods

Sampling and DNA Extraction

We collected 445 fresh tissue samples from 7 cohorts (2006–2011) (out of 530 turtles with microchips) by sampling the 3 existing captive populations of *B. trivittata* in Myanmar: Limpha Basecamp (Chindwin River) sampled in February 2013 ($n = 93$) and Yadanabon Zoological Gardens (Mandalay) ($n = 253$) and Lawkanandar Wildlife Sanctuary (Bagan) sampled in May 2013 ($n = 99$). Eighty-five turtles from the 2012 cohort were not sampled because of their small body size. We obtained tissue samples from the rear-foot webbing of each turtle and preserved them in absolute ethanol at 4 °C. We followed the Guidelines on the Care and Use of Animals for Scientific Purposes provided by the Singapore National Advisory Committee for Laboratory Animals Research (NACLAR) and National University of Singapore Institutional Animal Care and Use Committee (IACUC).

The DNA was extracted from the tissue samples following manufacturer's instructions for the DNeasy Blood and Tissue Kit (Qiagen, Hilden). The concentrations of DNA were measured with a dsDNA High Sensitivity Assay Kit (Qubit 2.0 Fluorometer, Invitrogen, Carlsbad).

Library Preparation and Sequencing of ddRAD-seq

Even though pedigree data were not available, we knew that most samples were half- or full-sibs. Therefore, we randomly subsampled 338 tissue samples to be processed for DNA library preparation. For these samples, we used the methods of Tay et al. (2016) to prepare 3 distinct ddRAD-seq libraries. The quality check of the final library fragment range was performed with a fragment analyzer automated capillary electrophoresis system (Advenced Analytical, Ankeny).

We sent the libraries to the Singapore Centre of Environmental Life Sciences Engineering at Nanyang Technological University, Singapore, for sequencing on an Illumina HiSeq 2500 platform with a paired end run in 3 lanes to yield maximum read lengths of 150 base pairs.

Figure 1. Location of Batagur trivittata *nesting (square) in the upper Chindwin River.*

SNP Identification and Validation

We checked data quality with FastQC version 0.11.3 (Andrews 2010). Raw data were demultiplexed in Stacks version 1.44 (Catchen et al. 2013) with process_radtags. We discarded sequences with a raw Phred score below 20, removed reads with uncalled bases or low-quality scores, and truncated reads to 140 bases to eliminate potential sequencing error occurring at the ends of reads. Only forward runs were analyzed because we wanted to mine unlinked markers and obtain only a single SNP per locus.

No reference genome is available for *B. trivittata*. Therefore, we assembled loci de novo with the denovo_map.pl program within Stacks (Catchen et al. 2013). This program builds loci for each sample, constructs catalog loci for specified populations, and matches individual loci to the catalog. We set the minimum depth of coverage (m) to 10, allowed 3 mismatches (M) in creating individual stacks and 2 mismatches in secondary reads (n), and removed highly repetitive RadTags. This parameter set yielded the highest number of biologically plausible SNPs. Once catalog loci and matches were identified, we ran the populations program in Stacks (Catchen et al. 2013) with a minimum of 90% of individuals in the population required to process a locus for that population. Six SNP data sets were generated, one with individuals with >10% missing data removed (scenario A) and

another with individuals with >20% missing data removed (scenario B). Three additional options for pruning SNPs with minor allele frequencies <0.05, <0.01, and 0 were applied for each scenario with Plink version 1.07 (Purcell et al. 2007). We then conducted a neutrality test for the resulting data sets with BayeScan version 2.1 (Foll & Gaggiotti 2008), which showed that none of the data sets included SNPs that significantly departed from neutrality. Mean polymorphic information content (PIC) of the SNPs was calculated with Cervus version 3.07 (Kalinowski et al. 2007).

Genetic Diversity and Population Structure

Observed heterozygosity (H_o), expected heterozygosity (H_e, gene diversity [Nei 1987]), and the inbreeding coefficient (G_{IT}) were calculated with GenoDive version 2.0b23 (Meirmans & Van Tienderen 2004). We used the methods-of-moments estimator in Coancestry version 1.0.1.7 (Ritland 1996; Wang 2011) to estimate inbreeding coefficients for each individual and calculated homozygosity by loci (HL) for each individual with the R function GENHET version 2.3 (Coulon 2010).

Because so few individuals remain in the wild and these turtles are potentially genetically related, we expected our data set to violate many assumptions of model-based population structuring, predominantly the assumption of unrelatedness (Anderson & Dunham 2008; Rodriguez-Ramilo & Wang 2012). Therefore, population structure was analyzed with NetView version 1.1 (Neuditschko et al. 2012; Steinig et al. 2016) in a network-based approach and with discriminant analysis of principal components (DAPCs) (Jombart et al. 2010) in a multivariate approach designed for clustering related individuals.

Mutual *k*-nearest neighbor graphs were created by applying NetView (Neuditschko et al. 2012; Steinig et al. 2016) for $k = 20$. The DAPC was performed with the package adegenet version 2.0.0 (Jombart 2008) in R (R Development Core Team 2015). The most likely genetic clusters in our data were determined with the find.clusters function. We retained 150 principal components that explain 90% of variation and used all discriminant functions to carry out the DAPC run.

Assurance and Reintroduction Colony Management

Our reintroduction objective was to have 2 groups of 30 individuals each (to be released at 2 different sites on the upper Chindwin River). An additional 25 individuals were selected to establish a captive assurance population. For each group, individuals with the lowest levels of inbreeding potential (as measured by HL) were selected from each of the genetic clusters. In this way, we aimed to select the most genetically diverse individuals in all genetic clusters to establish new breeding populations in the wild and in captivity.

Pedigree Analysis and Effective Population Size

Sibling analysis and pedigree reconstruction were performed using a very long run of the Full-Likelihood method in Colony (Jones & Wang 2010) in which female and male polygamy were allowed. A range of genotyping error rates was tested (10%, 1%, and 0.1%) for all loci. The noninbreeding model was selected and a sib-ship prior was not included. The only available information incorporated a priori in the program was the known maternal siblings (half-sibs) from the 2011 cohort (4 nests, 31 individuals [Supporting Information]). Parentage was accepted if the 3 error-rate runs provided the same parent dyads with posterior probability values of >90%.

To test whether the full-sib families could have resulted from chance alone, the relatedness among all samples in a family group was calculated using the Wang estimator (Wang 2002) in the R package related (Pew et al. 2015). Samples within each group were then randomly shuffled 1000 times with the grouprel function in related, and average expected relatedness values were compared with the observed ones.

Estimates of contemporary effective population sizes (N_e) were obtained for cohorts 2007–2011, but not for the captive breeders and their offspring, with the sibling assignment (SA) method (Wang & Santure 2009) available in Colony (Jones & Wang 2010). The 2006 cohort was not included in this analysis because of a small sample size ($n = 4$). We estimated N_e with the sibling assignment method because it does not assume random mating. The method assumes that a random sample of offspring was taken from a single cohort (as in our subsampling scheme) and that there are discrete nonoverlapping generations in the population (as is the case in the *B. trivittata* population). The number of breeders (N_b) was calculated on the basis of the inferred identity of parents of each individual from the 2007–2010 cohorts.

Results

We obtained sequence data for 338 samples and produced an average of 2.3 million (SD 0.8 million) reads per individual. We removed 21 samples, approximately 0.7% of all reads, owing to poor data recovery (<250 kb). We also eliminated individual samples with >10% missing loci.

The SNP pruning scenario B, which allowed for up to 20% missing data per individual, yielded more individuals ($n = 304$) than scenario A ($n = 276$), which allowed up to 10% missing data per individual. However, scenario A provided higher numbers of SNPs for each minor-allele-frequency setting relative to scenario B (Supporting

Table 1. Average homozygosity by loci (HL) values for each genetic cluster and each cohort group (2006–2011) of *B. trivittata*. Network cluster.

	Number of full-sib families	*Confirmed parents (female × male)* [a]	*Year* [b]								
			'06	*'07*	*'08*	*'09*	*'10*	*'11*	*unknown*	*Total*	*Average HL values*
1	1	1 × 1	0	11	14	11	28	11	15(1)	91	0.64
2	2	3 × 4 5 × 6	1(1)	4(3)	1	2	1(1)	0	1(4)	19	0.67
	2	C1 × C2 C2 × C1	0	0	0	6	12	0	8	26	0.50
3	1	2 × 3	2	17	0	1	0	2	2	24	0.63
4	1	2 × 2	0	1	4	12	31	10	15 (1)	74	0.67
5	1	6 × 7	0	7	9	5	26	8	15	70	0.65
		Total	4	43	28	37	99	31	62	304	
		average HL values of cohorts	0.61	0.65	0.65	0.65	0.68	0.66	NA		

[a] *Abbreviation: C, captive.*
[b] *Numbers in parentheses are the number of individuals for which parent sets were not identified.*

Information). We ran network analyses for these 6 different SNP data sets and observed 5 distinct genetic clusters identically distributed in each (Fig. 2 & Supporting Information), indicating that differences in SNP calling did not have major impacts on population-genomic results. Therefore, the data set that provided the highest number of individuals with the highest mean PIC (scenario B1 [Supporting Information]), which included 304 individuals with 1126 SNPs (mean PIC = 0.22), was used for all subsequent analyses.

Clusters 1 and 3–5 were composed of wild offspring only, whereas cluster 2 included captive breeders, their offspring (Fig. 2, Table 1), confiscated individuals, and some wild offspring. The DAPC yielded 6 clusters (Supporting Information) but differed from the network analysis only in that cluster 2 was represented by 2 combined clusters (Supporting Information).

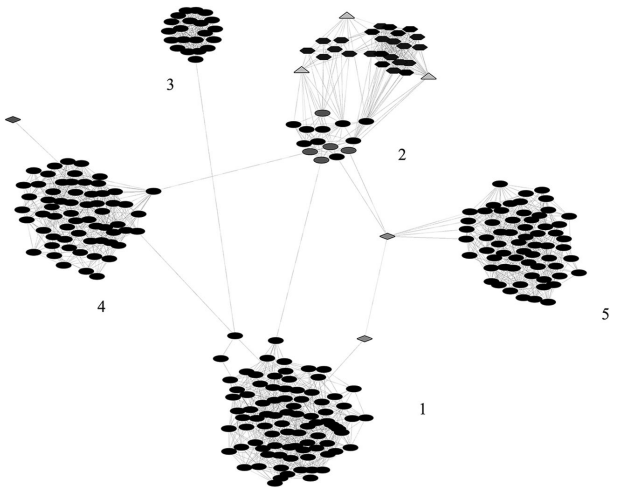

Figure 2. The 5 distinct clusters in the genetic similarity network of B. trivittata *(each shape indicates an individual (clusters 1 and 3–5, wild full-sib families; gray, unidentified parent sets; diamonds, confiscated Dokthawady individuals; triangles, captive-population founders; hexagons, captive offspring).*

The H_o (0.219 [SD 0.005]) did not significantly exceed H_e (0.213 [0.005]) (Fisher's exact test, $p = 1$), and G_{IT} was <0 (–0.027 [0.003]). HL values of all 304 samples ranged from 0.47 to 0.98, whereas individual inbreeding coefficients ranged from −0.26 to 0.45 (Table 1 & Supporting Information).

Parent sets were successfully assigned for 99.96% of individuals via SA analysis. Ten samples, including 3 captive breeders and 4 confiscated adults, had incompatible parent sets across different runs of Colony and 2 samples had <0.9 posterior probability assignments and were therefore excluded from further family assignment (gray shaded individuals, Fig. 2). Eight full-sib families were identified. Each network cluster provided 1 full-sib family, except cluster 2, which comprised of 2 wild and 2 captive (Fig. 2) full-sib families. By permuting the wild offspring data set, we found that the average relatedness within each full-sib group was significantly higher than a random distribution of relatedness values ($p < 0.001$) (Fig. 3). For each distinct cohort, N_b was greater than estimates of N_e (Table 2).

Discussion

Our data set included captive-reared progeny of all known breeding individuals (wild and confiscated) of the remaining global population of *B. trivittata*, and ours is one of few studies in which approximately half the global population of an endangered species was genomically sampled (e.g., Ryder et al. 2014). Therefore, we assumed the genetic diversity and structure we found directly reflected actual genetic diversity of the species globally. Although it may seem paradoxical that there was no sign of severe inbreeding (global $G_{IT} = -0.027$ [SD 0.003], Supporting Information) in either wild or captive born individuals of such a small population, our

Table 2. Estimated numbers of *B. trivittata* breeders (N_b) and the effective population size (N_e) for cohorts 2007–2011.

Cohort	N_b *(male, female)*	N_e *(95% CI)*
2007	8 (3, 5)	4 (2–12)
2008	7 (3, 4)	5 (2–20)
2009	10 (4, 6)	6 (3–21)
2010	7 (3, 4)	5 (2–20)
2011	5 (2, 3)	5 (2–20)

data set represented only the first generation of a population with a relatively recent bottleneck. Thus, the lack of severe signs of inbreeding should not be interpreted as a cause for relief because inbreeding coefficients would be expected to rise within the next few generations.

Genome-widedata can be used to pinpoint wild individuals contributing to the contemporary gene pool in a more powerful manner than traditional genetic markers (e.g., Allendorf et al. 2010; Avise 2010; Helyar et al. 2011). A prior estimate of <10 wild individuals was based on observational data (Platt et al. 2012, 2013), and the number of wild breeding males was unknown. Our estimates of the number of wild female ($n = 2$) and male ($n = 3$) breeding adults (as of 2011) (Table 2) were much lower, indicating a more dire situation than previously recognized. The N_e remained relatively stable from 2007 to 2011 (Table 2), albeit alarmingly low, rendering the population highly vulnerable to demographic and genetic stochasticity (Frankham 2005).

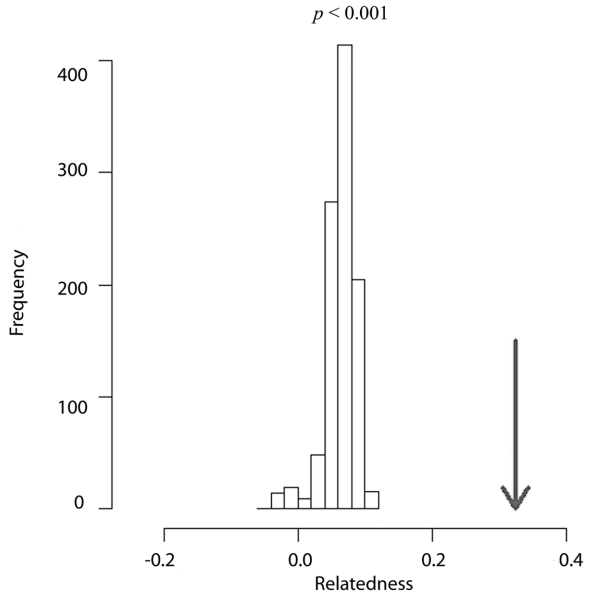

Figure 3. Expected relatedness values within each full-sib family of B. trivittata *(arrow, observed relatedness value).*

Our network and DAPC analyses suggest that each of the 4 genetic clusters (Fig. 2 & Supporting Information) comprises one full-sib family with distinct fathers for each family except for group 2. Group 2 represented primarily confiscated individuals from the Chindwin River ($n = 3$) and their captive-born offspring ($n = 28$). Another 3 individuals from the founder colony, which were confiscated or rescued from the Dokthawady River system, have not started breeding in captivity and clustered with groups 1, 4, and 5, albeit not closely (Fig. 2). Some of the original wild males stopped contributing to the gene pool over the study period (2007–2011), suggesting their potential death. This assumption is consistent with the decreasing number of wild-collected fertile eggs (2012, 87; 2013, 53; 2014, 1; 2015, 0) and indicates a severe reduction in reproductive success in the wild, likely due to a lack of free-ranging males (Platt et al. 2015).

Following the sharp recent declines in wild breeding activity and the success of captive breeding, approximately 99% of the current >700 individuals of *B. trivittata* live in captivity. Given the population is highly genetically and demographically impoverished, management should focus on retaining contemporary genetic diversity and increasing the number of individuals in the wild. To this end, a fine-scale assessment of population structure and pedigree analysis plays a key role in selecting ideal individuals for reintroduction efforts and assurance colony management. Accordingly, 2 groups of 30 individuals were selected based on our results and released in (February 2015) 2 places along the upper Chindwin River, where active nesting beaches exist (Platt et al. 2014, 2015). In the 2015–2016 breeding season (postrelease), we obtained 27 fertile eggs from the known nesting beaches in the wild, likely because the released males contributed to active breeding output, which had previously shrunk to zero in 2015. A captive satellite colony of 25 individuals—directly selected on the basis of our population-genomic data to maximize genetic diversity—was transferred to the Singapore Zoo in May 2016 (S. Luz, personal communication) so a genetic stock for the species would be secured outside of Myanmar. The current captive pool includes founders collected from the Dokthawady River that are relatively unrelated to all other known individuals. Once they start breeding, overall genetic diversity within the captive offspring population should be considerably improved. We used genomic methods to inform choices for reintroduction- and assurance-colony management. Our results may help preserve the genetic diversity of *B. trivittata* in the wild, and our study is illustrative of how the gap between in situ and ex situ conservation can be bridged.

The number of conservation genomic studies has grown (Shafer et al. 2015), and the results of these studies are being applied on the ground (Garner et al. 2016). However, there remains a lack of case studies carried

out on nonmodel and noncommercial species (Shafer et al. 2016). We believe our work shows how to fill this scientific gap generally and with regard to one of the most critically endangered and enigmatic turtle species by shedding light on genomic variation, population structure, and demographic parameters of the last remaining individuals. Furthermore, a research-to-application gap exists in conservation genomic studies (Shafer et al. 2015) that we bridged through our use of genomic approaches to guide real-world reintroduction and ex situ management of a critically endangered species.

To our knowledge ours is the most extensive sampling scheme for a genomic study conducted with vertebrates, except for the Californian Condor (*Gymnogyps californianus*) genomics project (Ryder et al. 2014) in which all the genomes of all known populations of the species were sequenced. There are many other species with similar extirpation histories that a genomic approach could be applied to. We showed that addressing pressing conservation problems is feasible with genome-wide SNP screening and suggest this method become the conservation genetic standard for the midterm future.

Our study has substantial implications for a robust conservation program in 2 distinct ways. First, it provides a better understanding of the current status of *B. trivittata* in the wild because we provided estimates of the numbers of wild breeders and determined current genetic structure. Second, our results directly affected the future genetic diversity of *B. trivittata* because they informed the genetic and demographic reinforcement of the wild population and management of the assurance colonies outside Myanmar. We believe our research provides a pioneering case study of bridging in situ and ex situ conservation through the use of genome-wide DNA data.

Acknowledgments

We are grateful to the Myanmar Ministry of Natural Resources and Environmental Conservation, the Wildlife Conservation Society, and the Turtle Survival Alliance for their support during the study. Burmese roofed turtle conservation within Myanmar was funded by A. Sabin and The Andrew Sabin Family Foundation, Panaphil Foundation, Helmsley Charitable Trust, and the Critically Endangered Ecosystem Partnership Fund. The genomics part of the study was funded by Wildlife Conservation Society, Loke Cheng-Kim Foundation, the Phoenix Zoo, Wildlife Reserves Singapore, and a Singapore Ministry of Education Tier I grant (WBS R-154-000-691-112). We thank B. Chattopadhyay for his valuable comments on the bioinformatic analyses. We also thank B. Horne, C. Poole, S. Luz, and G. Kuchling for their support and feedback during the study.

Supporting Information

A photograph of an adult male of *Batagur trivittata* with breeding pattern (courtesy of Rick Hudson, Turtle Survival Alliance) (Appendix S1), the list of 304 samples used and the nesting data from cohort 2011 (Appendix S2), the summary of 6 different SNP data sets (Appendix S3), similarity networks for these 6 data sets (Appendix S4), Bayesian information criteria for different numbers of DAPC clusters (Appendix S5), the DAPC plot of our data set (Appendix S6), and HL values of the 304 samples and assurance and reintroduction colony assignments (Appendix S7) are available online. The authors are solely responsible for the content and functionality of these materials. Queries (other than absence of the material) should be directed to the corresponding authors.

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