A Preliminary Inbreeding Study of Malaysian Endangered Southern River Terrapin (*Batagur affinis*) from Captive Population of Botakanan, Perak and Wild Population of Kemaman, Terengganu

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Abstract: The effects of inbreeding are important in conservation genetic studies of endangered animals. Inbreeding can leads to inbreeding depression due to low level of genetic diversity in a population. Consequently affect population growth and viability. Hence, it is a major concern in many conservation programs of endangered animals to avoid the effects of inbreeding and at the same time maintain the genetic diversity. To date, information about inbreeding and genetic diversity can be assessed using molecular markers such as microsatellites. In this study, a preliminary inbreeding information of Malaysian endangered Southern river terrapin (*Batagur affinis*) from two populations: (i) BotaKanan, Perak (captive population), and (ii) Kemaman, Terengganu (wild population) were studied using nine cross-species microsatellites. It was found that inbreeding was higher in BotaKanan population than Kemaman population (F_{is} = 0.57 *c.f.*F_{is} = 0.43). BotaKanan population also carried lower genetic diversity than Kemaman population (H_O = 0.29 *c.f.*H_O = 0.40). Hence, suggests that including genetics information to improve the current breeding program and further genetics study to access various life-history traits in BotaKanan population is necessary. In addition, study to estimate effective population size and to access various life-history traits are also needed for Kemaman population to avoid inbreeding in the near future.

1 INTRODUCTION

In conservation genetics, preservation of maximum genetic diversity (minimisation of inbreeding or co-ancestry or kinship) is an important factor to ensure the perseverance and evolution of endangered, small sized population (i.e. small effective population size) and isolated species (Brook *et al*, 2002; Blambert *et al,* 2016), particularly due to changes in environmental conditions — mainly under stress conditions (e.g. Hendrick and Kalinowski, 2000; Frankham, 2003). Reduced genetic diversity may negatively impact the adaptive potential for endangered, small and isolated species, because alleles are randomly fixed or lost from the species by drift. Hence, deleterious mutations tend to accumulate. However, the process is rather slow, and thus, does not reduce growth and increase extinction rates in the short term. On the other hand, inbreeding effect is rather severe and immediate in small sized and isolated species, especially for species which experienced bottlenecks (Barrett and Charlesworth, 1991; Saccheri *et al*, 1996). Small and isolated species tend to fix considerable fraction of genetic load by increasing the frequency of individuals with homozygous for alleles identical by descent (Witzenberger and Hochkirch, 2011), and increasing the uncovering of deleterious recessive alleles (Witzenberger and Hochkirch, 2011; Nielsen and Slatkin, 2013; Franham *et al*, 2010). These result in fitness reduction (i.e. inbreeding depression) which are uncover through reduce offspring survival (Coltman *et al*, 1998; Amos *et al*, 2001), reduce fertility, decrease mating success (Alados and Escos, 1991), slow development, increase sterility and increase susceptibility to environmental/disease/ parasite stress (Coltman *et al*, 1999; Cassinello *et al*, 2001; Keller and Waller, 2002; Ambruster and Reed,

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2005; Boakes *et al*, 2007; Charlesworth and Willis, 2009). Furthermore, there is positive association between inbreeding and extinction, particularly when the growth rate of the species is low (e.g. house fly (Byant and Meffert, 1990); Drosophila and the house mouse (Frankham, 1995); *Bicyclus anyana* (Saccheri *et al*, 1996); *Clarkia pulchenella* (Newman and Pilson, 1997); *Drosophila* (Bijlsama *et al*, 2000)). Hence, justify the importance to access inbreeding in endangered, small sized and isolated species.

To access inbreeding, information from studbook record and/or molecular genetics data can be used (Pemberton, 2004). The studbook record usually comprises of basic information such as birth and death dates, weight, identification number, translocation record and selected life-history traits with incomplete pedigree or questionable paternity assignment data (usually breeding individuals and their offspring) of the breeding population/species. Hence, inbreeding can be significantly under or overestimate based on studbook data alone (Ito *et al*, 2017; Willis, 2001). The pedigree data from studbook record can only be used to calculate inbreeding coefficients (F) based on the assumption that founders are unrelated and non-inbred, and individuals of unknown origin have a high level of relatedness. Meanwhile, molecular genetics analysis can provides much more realistic inbreeding assessment. It measures the inbreeding (F_{is}) based on the deviation of the observed heterozygote relative to the expected heterozygote under random mating (Hardy-Weinberg equilibrium). Using this method, highly polymorphic genetic markers such as Single Nucleotide Polymorphisms (SNPs), microsatellites (SSRs; Simple Sequence Repeats; e.g. (Solkkoe and Toonen, 2006)), Amplified Fragment Length Polymorphism (AFLP; e.g. (Gardiner *et al*, 2017)), and Random Amplified Polymorphic DNA (RAPD) are use. Among those markers, microsatellite is the most commonly used (Solkkoe and Toonen, 2006). This is due to it is a DNA marker which can provides high information content for small sized or recently bottlenecked species (Hedrick, 1999).

In Malaysia, Malaysian Southern river terrapin (*Batagur affinis*; Figure 1) which is one of endangered species (UCN, 2018) have been conserved and protected by government (Department of Wildlife and National Park (PERHILITAN)) and non-government (Turtle Conservation Society of Malaysia (TCS)) agencies of Malaysia. Three conservation centres at three states (i.e. Perak, Terengganu and Kedah) were built. Those conservation centres are: (i) the BotaKanan Conservation Centre, Perak, (ii) the Terrapin Conservation Centre, Kemaman, Terengganu, and (iii) the Wildlife Conservation Centre, Bukit Pinang, Kedah. Those conservation centres are focusing on captive breeding and/or raising hatchlings (Moll *et al*, 2015). Most hatchlings are releaseas head-start hatchlings to wild populations with aim to increase the numbers of individuals'*B. affinis* in the wild populations. However, the numbers of individuals in the wild populations are kept on declining (Moll *et al*, 2015). There are currently known non-biological factors including illegal meat and eggs trading as an exotic delicacy, sand mining activities at its nesting river banks and aquaculture activities along its residential rivers that cause the decline number of individuals in the wild populations (Moll *et al*, 2015). However, to our awareness, no genetic factors including inbreeding had been reported for *B. affinis*. Therefore, a preliminary study to understand inbreeding which is important genetic information using cross-amplified microsatellite markers was conducted to access inbreeding and genetic deversity in two populations of *B. affinis*: (i) BotaKanan, Perak (captive population), and (ii) Kemaman, Terengganu (wild population).

Figure 1: A female Malaysian Southern river terrapin (*Batagur affinis*).

2 MATERIALS AND METHODS

Tissue sample collection

Two populations of *B. affinis* were used in this study: the captive population of BotaKanan, Perak and the wild population of Kemaman, Terengganu (Figure 2). A total of 30 pieces of tissues from the swimming web of the rear foot of randomly chosen adults were sampled from the BotaKanan Conservation Centre, Perak. Whilst a total of 30 pieces of tissues from the sculte of randomly chosen hatchlings were sampled from the Kemaman River Terrapin Conservation Centre, Terengganu. All tissues were collected with the help of staffs at each conservation centre under special permit (T-00465-16-16) and NRE 600-2/2/21 Jld.4 (12). Those tissues were kept in separate 1.5 ml microcentrifuge tube containing 70% ethanol at room temperature.

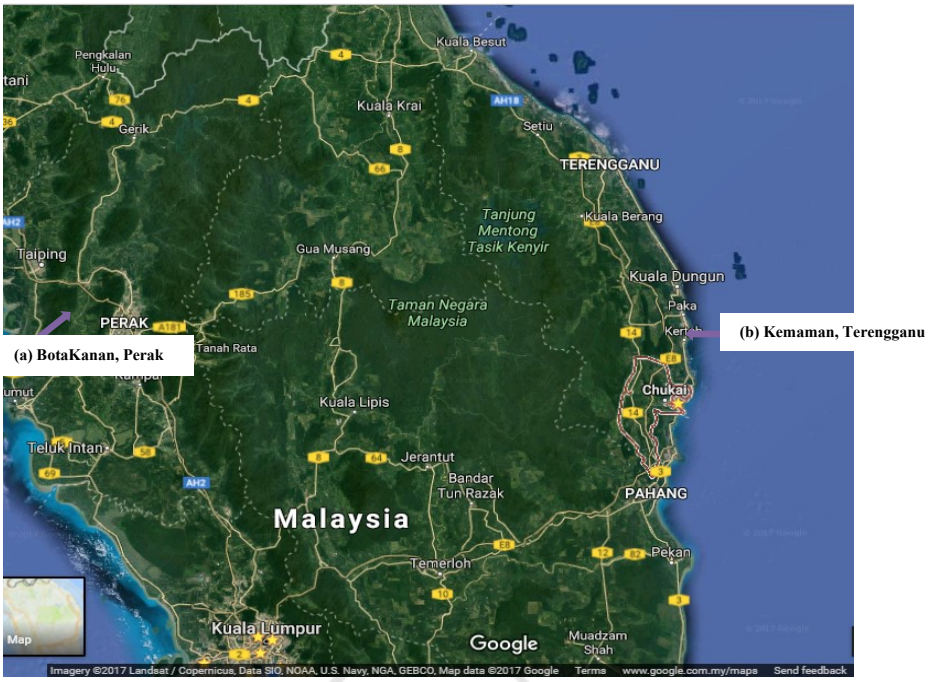

Figure 2: Two sampling populations of *B. affinis*: (a) the captive population of BotaKanan, Perak, and (b) the wild population of Kemaman, Terengganu. (Source: Google Satellite, 2018).

Data collection

All DNA was isolated using ReliaPrep™ gDNA Tissue Miniprep System kit by following the manufacture protocol. All DNA extractions were eluted in 100 µl of Nuclease-Free water and kept at -20ºC. Then, Polymerase Chain Reactions (PCRs) were carried out using nine cross-species microsatellites (four microsatellites of Burmese Roofed Turtle [*B. trivittata*]: (Love *et al*, 2013), and five microsatellites of Yellow-headed Sideneck [*Podocnemis unifilis*]: (Fantin *et al*, 2007; Table 1) to obtain genotyping data for inbreeding study. The following was PCR protocol of *B. trivittata* microsatellites: Pre-denature at 95ºC for 4 min. Followed by 30 cycles of denaturation at 94ºC for 1 min, annealing ranging from 50 to 65 ºC for 1 min, extension at 72ºC for 1 min and final extension at 72ºC for 20 min. Whilst the PCR protocol of *P. unifilis* microsatellites were performed as followed:initial denaturation at 94ºC for 1 min, 45 cycles of denaturation at 92ºC for 50 sec, annealing temperature ranging from 50 to 70ºC for 1 min, extension at 72ºC for 1 min, and final extension for 20 min. The PCRs volume for all nine cross-amplified microsatellites (hereafter loci) contained 3 µl of DNA template, 0.7 µl of $MgCl_2$, 1.0 µl of buffer, 1.2 µl of Taq polymerase, 0.8 µl of dNTPs mix, 0.6 µl of each forward and reverse microsatellite primers and 2.1 µl of ddH_2O. The PCR products were then visualized on 3% agarose gel electrophoresis and run for 90 min at 78 V with 20 bp size marker as allele size reference to obtain genotyping data by manually scored.

All genotyping data were checked using MICROCHECKER (van Oosterhout *et al*, 2004) for null alleles or allelic dropout. Then, CREATE software (Coombs *et al*, 2008) was used to convert the genotyping data into a readable format for inbreeding and genetic diversity analysis. Finally, inbreeding (F_{is} value: (Weir and Cockerham, 1984)) and genetic diversity (H_O) in the two populations were calculated using GENEPOP on the web (http://genepop.curtin.edu.au/).

3 RESULTS

Generally, the Malaysian *B. affinis* from wild population of Kemaman population comprised of less inbred individuals as compared to captive population of BotaKanan population (F_{is} = 0.43 *c.f.* F_{is} = 0.57; Table 1). This was showed from all of nine cross-amplified loci, Kemaman population had four loci with positive F_{is} value (Table 1). Whilst BotaKanan population had seven loci with positive F_{is} value (Table 1). The less inbred Kemaman population also showed higher level of genetic

diversity than BotaKanan population (H_O = 0.40 *c.f.* H_O = 0.29). The Kemaman population also showed three loci with observed heterozygote value of more than 0.50 (H_O> 0.50: Puni_1E1, Puni_1H9 and Puni_2C11; Table 2). Whilst the BotaKanan population only showed two loci (H_E> 0.50: Puni_1H9 and Puni_2A9; Table 2). However, both populations showed two out of the nine tested loci were in heterozygote deficit (H_O = 0.00; Table 2). With both showed similar heterozygote deficit at one locus (Puni_1A5), but, at locus Puni_2A9 for Kemaman population and Puni_1E1 for BotaKanan population. In addition, BotaKanan population had lower value of observed heterozygosity than expected heterozygosity (mean H_O = 0.29 <H_E = 0.46; Table 2). Whereas Kemaman population had slightly higher value of observed heterozygosity than expected heterozygosity (mean H_O = 0.40 >H_E = 0.39; Table 2).

Table 1: The F_{is} values of all nine cross-amplified loci for Kemaman and BotaKanan populations of Southern river terrapin.

Locus	F_{is}value	
	Kemaman	**BotaKanan**
Batr16	0.89	0.51
Batr25	0.49	0.99
Batr30	0.98	0.28
Batr39	0.32	0.91
Puni_1A5	-	-
Puni_1E1	-0.52	1.00
Puni_1H9	-0.07	0.57
Puni_2A9	-	-0.41
Puni_2C11	-0.87	0.28

Table 2: The observed heterozygosity values (H_O) and expected heterozygosity values (H_E) of all nine cross-amplified loci for Kemaman and BotaKanan populations of Southern river terrapin.

Locus	Kemaman		BotaKanan	
	H_O	H_E	H_O	H_E
Batr16	0.38	0.60	0.40	0.65
Batr25	0.31	0.39	0.03	0.35
Batr30	0.03	0.47	0.45	0.53
Batr39	0.30	0.57	0.05	0.52
Puni_1A5	0.00	0.00	0.00	0.00
Puni_1E1	0.70	0.46	0.00	0.45
Puni_1H9	0.97	0.54	0.57	0.65
Puni_2A9	0.00	0.00	0.60	0.43
Puni_2C11	0.93	0.51	0.50	0.58
Mean	**0.40**	**0.39**	**0.29**	**0.46**

3.1 Discussion

B. affinis can be found at three states of Malaysia: Kedah, Perak and Terengganu. In this preliminary inbreeding study, using nine cross-amplified microsatellites, two populations of *B. affinis* were selected (captive population of BotaKanan, Perak and wild population of Kemaman, Terengganu). It was confirmed that Kemaman population had lesser loci with positive F_{is} value as compared to BotaKanan population (Kemaman population: four loci *c.f.* BotaKanan population: seven loci). This suggests that the wild population of Kemaman, Terengganu was less inbred as compared to the captive population of BotaKanan, Perak (F_{is} = 0.43 *c.f.* F_{is} = 0.57). In addition, the result of genetic diversity (H_O) supported the finding of inbreeding (F_{is} value) in this study. It was showed that the less inbred wild population of Kemaman had higher genetic diversity than captive population of BotaKanan (H_O = 0.40 *c.f.*H_O = 0.29).

Higher inbreeding and number of loci with positive F_{is} values in captive population of *B. affinis* from BotaKanan as compared to wild population of Kemaman suggests that mating between siblings is likely high among individuals in BotaKanan. This is probably due to the current captive population of BotaKanan that consists of about 200 adult *B. affinis*. But, detail pedigree record either from studbook data and/or molecular analysis of those 200 adults is not available. Hence the probability of identical by descent among those 200 adults cannot be clearly determined. Though, the result of F_{is} values from this study clearly suggests that there is probability of at least 30 from those 200 adults are inbreed. All of those adults were captured from the nearby Perak River and keep in two main ponds (known as Pond A and B) at the BotaKanan River Terrapin Conservation Centre, BotaKanan, Perak. In addition, among those 200 adults, not all of them are fertile (N_e: genetic effective population size; personal conversation with the staffs of PERHILITAN Perak). Hence, it is necessary to further study and improves the current conservation program of *B. affinis* at BotaKanan. Apparently, there is no assurance about the appropriate number of individuals to be kept for ensuring the persistence of a population (Berger, 1990). Yet, the conservation genetics proposes the '50/500' rule. Suggesting the genetic effective population size should be maintained above 50 at all times to avoid inbreeding, and above 500 to retain evolutionary potential (Witzenberger and Hochkirch, 2011;Harmon and Braude, 2010). Because these values would tolerate

the effect of random drift to fix deleterious alleles in short term (i.e. inbreeding depression), whilst maintaining genetic diversity to minimise the effect of selection over long term (Harmon and Braude, 2010). However, the implementation of '50/500' rule depends on the number of founders and population history such as a population's network (Harmon and Braude, 2010). On the other hand, the inbreeding situation of *B. affinis* from Kemaman population is perhaps less urgent than is the case of *B. affinis* from BotaKanan, which probably consist of more genetic effective population size than the captive population of BotaKanan. Though the current exact number of genetic effective population size, and detail pedigree record either from studbook data and/or molecular data is not available for Kemaman population because of no such study had been conducted.

In addition, the lower genetic diversity (referring to heterozygosity) in captive population of BotaKanan as compared to the wild population of Kemaman had supported the high inbred result. Low genetic diversity has also been reported in several threatened species elsewhere in the world (e.g. (González-Pérez *et al*,2004; Spitzweg *et al*, 2018). The reasons contribute to this are population size and variability (Nielsen and Slatkin, 2013;González-Pérez *et al*,2004; Spitzweg *et al*, 2018). Low genetic diversity in the captive population of BotaKanan probably due to this population had been started with either low number of founders (bottleneck) or highly associated founders (i.e. maximisation of the effective population size). Both number and association of founders are major factors determining the gene pool. Low number of founders can significantly increase the accumulation of deleterious alleles and consequently lead to inbreeding depression (Witzenberger and Hochkirch, 2011; Nielsen and Slatkin, 2013; Franham *et al*, 2010). Whist highly associated founders increase the chance for inheriting homozygous alleles at each locus because of the same alleles are passed to offspring from common ancestors (Witzenberger and Hochkirch, 2011). Witzenberger and Hochkirch, 2011 proposed that a minimum of 15 founders of census population size (N_c) seemed to be sufficient to maintain good genetic diversity to minimise inbreeding. Whereas Franham *et al*, 2010 proposed that 20 – 30 founders of genetic effective population size were needed to maintain good genetic diversity to minimise inbreeding. In conservation genetics, preservation of maximum genetic diversity (minimisation of inbreeding or co-ancestry or kinship) is an important factor to ensure the perseverance and evolution of endangered, small sized (i.e. small effective population size) and isolated species (Brook *et al*, 2002; Blambert *et al,* 2016), particularly due to changes in environmental conditions — mainly under stress conditions (e.g. (Hendrick and Kalinowski, 2000; Frankham, 2003)).

In this preliminary study of *B. affinis*from BotaKanan and Kemaman populations, only molecular data was used to access the inbreeding and genetic diversity information. Though the acquired information was sufficient to suggest the present of higher inbreeding and lower genetic diversity in BotaKanan population as compared to Kemaman population, more detail study regarding this matter would be necessary to improve the current conservation programs of Malaysian *B. affinis*. Combination of molecular data and studbook data to measure relationships between individuals will provide better conservation genetics information (e.g. inbreeding, genetic diversity, life-history traits, demographic and stochastic trends, and etc.) in small sized population, isolated and recently bottlenecked species (Pemberton, 2004). The Malaysian *B. affinis* is a small sized population species due to its low turnover (i.e. 25 years old to reach age of maturation, or earlier at 22 years old at carapace size of 510 mm, annually reproduce and 23 to 30 of average number of eggs per clutch (Chan and Chen, 2011), isolated because it is a fresh water species with restricted pattern of distribution, and probably experienced recent bottleneck due to environmental change in their local habitat — including the effects of sand mining, dam construction and aquaculture activities (Moll *et al*, 2015). By including molecular data, reliable information about allele distribution (i.e. assessing the frequency of heterozygous, homozygous dominant and homozygous recessive alleles) which evident for genetic diversity that significantly relates to inbreeding, as well as life-history traits, demographic and stochastic trends can be assessed. Also, molecular data can provide more accurate pedigree information than studbook data. Whereas by including studbook data, inbreeding coefficients (F) based on the assumption that founders are unrelated and non-inbred, and individuals of unknown origin have a high level of relatedness information can be evaluated. Hence, including conservation genetics information in the current conservation programs of Malaysian *B. affinis* (i.e. conventional conservation practice) will help to improve the population size of this species in the wild populations, as well as in the captive populations.

4 CONCLUSION

In conclusion, mating between relatives (inbreeding) is high in captive population of BotaKanan, Perak as compared to the wild population of Kemaman, Terengganu. Hence, further conservation genetic studies, combining both molecular data and studbook data should be conducted at the BotaKanan, Perak population to access better genetic information, particularly information of inbreeding and life-history in the current population. Whereas further molecular, parentage and life-history studies in the Kemaman, Terengganu population should be estimated to ensure inbreeding is not a problem in the near future.

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