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Conservation genetics of the northern river terrapin (*Batagur baska*) breeding project using a microsatellite marker system

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Abstract. The drastic decline of the critically endangered northern river terrapin (*Batagur baska*) prompted a large-scale captive breeding project in Bangladesh and Austria, with the first captive-bred offspring in 2010. Initially, males and females were kept together and mated without any system. However, controlled breeding was desired to conserve genetic diversity. For revealing relationships among the adult breeding stock and parentages of juveniles, we established a powerful genetic marker system using 13 microsatellite loci. Our results indicate that most wild-caught adults of the breeding groups are related, suggesting that the wild populations experienced a severe decline long time ago. We develop recommendations for breeding to preserve a maximum of genetic diversity. In addition, we provide firm genetic evidence for multiple paternity and sperm storage in *B. baska*. Our microsatellite marker system is promising to be useful in breeding projects for the other five *Batagur* species, which are all considered to be Critically Endangered or Endangered. We recommend implementing conservation genetic assessments for captive breeding projects of turtles on a broader scale to preserve genetic diversity and to avoid inbreeding.

Key words. Bangladesh, captive breeding, conservation, Critically Endangered, India, pedigree.

Introduction

The northern river terrapin *Batagur baska* (Gray, 1830) is a large riverine and estuarine turtle species with a carapace length of up to 60 cm (Moll et al. 2009). Like the other five *Batagur* species, the northern river terrapin is characterized by a species-specific, conspicuous breeding coloration in males, which seems to play an important role as a prezygotic isolating mechanism (Praschag et al. 2009). In *B. baska*, head and neck of breeding males are black, their forelimbs and dorsum of the neck range from light orange to rich crimson, and the iris appears yellow (Fig. 1). Females are generally distinctly less colourful and are, for some species, morphologically difficult to tell apart (Anderson 1878, Moll et al. 2009, Praschag et al. 2009, unpubl. observ.). According to reports of fishermen in the Sundarbans, males of *B. baska* are typically caught in estuaries and along the surrounding coastline, while females are found more upstream, especially during the nesting season (Moll et al. 2009).

For a long time, *B. baska* was thought to be distributed from northeastern India through the Malay Peninsula to Sumatra and Cambodia (Iverson 1992). However, it was later discovered that the southern and eastern populations represent a distinct species, the southern river terrapin *Batagur affinis* (Cantor, 1847) (Praschag et al. 2007, 2008, 2009). *Batagur baska* sensu stricto historically occurred from Odisha and West Bengal (India) through Bangladesh to at least the Ayeyarwady and Bago river mouths in Myanmar, and possibly to the estuaries of the Thanlwin and Sittaung rivers in this country. However, *B. baska* has been extirpated from most of its former range with no known wild populations remaining (Praschag et al. 2008, Moll et al. 2009). It is now regarded as one of the 25 most critically endangered turtle species of the world (Rhodin et al. 2011) and is considered ecologically extinct (Weissenbacher et al. 2015). The species is currently included in CITES Appendix I and classified as 'Critically Endangered A1cd' by the IUCN (Rhodin et al. 2017).

During the early 1990s, attempts to preserve river terrapins now assigned to *B. baska* sensu stricto failed because of political unrest (Moll et al. 2009). Conservation efforts focused on populations in Malaysia, then thought to be conspecific. After the recognition of the northern river terrapin as a species distinct from *B. affinis*, efforts were resumed, but conservationists needed years to discover a

few live terrapins in fish breeding ponds and markets. The first individuals were transferred for captive breeding to the Turtle Island facility in Graz and the Vienna Zoo, Austria, where two juveniles hatched in 2010. For large-scale breeding, the 'Project Batagur' was later launched with 14 males and six females in the Bhawal National Park (Bangladesh), as a joint initiative of the Bangladesh Forest Department, the Turtle Survival Alliance (TSA), the Vienna Zoo, and the IUCN Bangladesh (Weissenbacher et al. 2015). First breeding occurred there in 2012 and 2013. However, because of the limited number of breeders, a strategy was needed to preserve as much genetic diversity as possible. To ascertain the relationships among the founders and establish such a strategy, we genotyped all northern river terrapins from the breeding projects using 13 microsatellite loci. Based on this data, we analysed their relationship and developed recommendations for the combination of breeding groups.

Materials and methods

Sampling

Currently, 14 males, six females and three wild-caught juveniles are kept in the 'Project Batagur' facilities in Bangladesh. Another wild-caught pair and the two first captive-bred juveniles (males) live at Vienna Zoo; another male, two females and three juveniles are housed at Turtle Island, Graz. Blood and tissue samples of all terrapins were studied. An additional male from the project in Bangladesh could not be included because it died in 2013, before terrapins were sampled. In addition to the adult terrapins, 23 juveniles from four nests (breeding season 2012) and 61 juveniles from another four nests (breeding season 2013) were sampled (Tables S1 and S2). Samples were preserved in pure ethanol and kept at -20°C until processing. All terrapins in Bangladesh are microchipped, allowing individual identification.

Laboratory procedures

Total DNA was isolated using the InnuPrep DNA Mini Kit for tissues and the InnuPrep Blood DNA Mini Kit for blood samples (both kits: Analytik Jena GmbH). Since no specific primers for microsatellite loci of any *Batagur* species were available, a wide range of primers developed for other turtle species was tested for cross amplification (Supplementary Table S3). The presence of the microsatellites in the amplicons was confirmed by sequencing in both di-

Figure 1. Adult male of *Batagur baska* in breeding coloration. Photo: Peter Praschag.

Table 1. Genetic diversity of studied *Batagur baska*. n – sample size, nA – alleles per locus, AR – allelic richness, H_O – observed heterozygosity, H_E – expected heterozygosity, p – probability, HWE – Hardy-Weinberg equilibrium.

	Wild-caught terrapins									Captive-bred terrapins from Bangladesh								
Locus	n	nA	Private alleles	AR	H_O	H_E	p	HWE	Null alleles	n	nA	Private alleles	AR	H_O	H_E	p	HWE	Null alleles
GP19	33	8	2	7.752	0.636	0.772	0.530	yes	no	83	6	–	5.981	0.783	0.712	0.422	yes	no
GmuB08	29	4	–	4.000	0.724	0.603	0.643	yes	no	81	6	–	5.877	0.444	0.484	0.743	yes	no
Test21	33	5	1	4.817	0.515	0.622	0.338	yes	no	83	4	–	4.000	0.723	0.637	0.488	yes	no
TWS190	32	3	1	2.844	0.594	0.496	0.441	yes	no	83	2	–	2.000	0.518	0.398	0.005	no	no
TWT113	32	2	–	2.000	0.719	0.468	0.002	no	no	78	2	–	2.000	0.833	0.489	0.000	no	no
M01	32	3	1	2.844	0.344	0.446	0.128	yes	no	80	2	–	2.000	0.413	0.418	1.000	yes	no
M18	33	14	4	13.262	0.879	0.900	0.440	yes	no	80	10	–	9.981	0.850	0.841	0.000	no	no
M03	33	2	–	2.000	0.152	0.193	0.298	yes	no	83	2	–	2.000	0.205	0.257	0.078	yes	no
M24	33	7	2	6.813	0.727	0.795	0.420	yes	no	84	5	–	5.000	0.714	0.704	0.000	no	no
M06	33	10	4	9.539	0.788	0.752	0.610	yes	no	84	6	–	5.857	0.702	0.682	0.000	no	no
M21	33	13	5	12.240	0.879	0.879	0.714	yes	no	83	8	–	7.867	0.867	0.836	0.000	no	no
msEo41	33	3	–	2.969	0.364	0.386	0.777	yes	no	83	3	–	3.000	0.446	0.477	0.024	no	no
GmuD16	27	14	5	14.000	0.852	0.891	0.674	yes	no	72	9	–	9.000	0.806	0.843	0.004	no	no
Mean	32	6.769		6.545	0.629	0.631				81.31	5.000		4.966	0.645	0.602			
SD		4.423		4.248	0.218	0.214					2.689		2.667	0.196	0.182			

rections using an ABI 3730 DNA Analyzer (Applied Biosystems), and allele size ranges were examined to identify informative loci. This resulted in the selection of 13 loci.

To amplify microsatellite DNA, nine multiplex PCRs were performed using a reaction volume of 10 µl containing 10–20 ng of total DNA, 0.5 units of Taq polymerase (Bioron), the buffer recommended by the supplier, 1.5 mM $MgCl_2$ (Bioron), 0.2 mM of each dNTP (Thermo Scientific) and 2 µg of bovine serum albumin (Thermo Scientific). In addition, each multiplex PCR comprised a specific set of primers at a specific concentration.

Multiplex 1 contained for the loci GmuD51, GP19 and Test56 0.3 µM of each primer; multiplex 2 contained GmuB08 (0.3 µM) and GP55 (1.0 µM); multiplex 3 contained Goag5, GP81 (0.5 µM each) plus GP61 and Test21 (0.3 µM each); multiplex 4 contained TWL61, TWS190 and TWT113 (0.3 µM each); multiplex 5 contained Maucas01 (0.3 µM), Maucas18 (0.5 µM) and Maucas22 (0.6 µM); multiplex 6 contained Maucas03 (0.6 µM) and Maucas17 (0.3 µM); multiplex 7 contained Maucas06 and Maucas24 (0.3 µM each); multiplex 8 contained Maucas05, Maucas14 and Maucas21 (0.3 µM each), and multiplex 9 contained GmuD16 and GmuD55 (0.3 µM each) plus msEo41 (0.5 µM). Thermocycling conditions are summarized in Supplementary Table S4. Fragment lengths were determined using an ABI 3730 DNA Analyzer. For this purpose, 1 µl of the PCR product was diluted in 99 µl water; 1 µl of this dilution was mixed with 8.5 µl Hi-Di Formamide (Applied Biosystems), 0.25 µl water and 0.25 µl GeneScan-600 LIZ Size Standard (Applied Biosystems). The software PEAK SCANNER 1.0 (Life Technologies, Carlsbad, CA) was used for scoring fragment lengths. GmuD51, GP55, Maucas05, Maucas14, Maucas22, Test56, and TWL61 turned out to be monomorphic, whereas GmuD55, Goag5, GP61, GP81, and Maucas17 had an overrepresentation of homozygotes, therefore only the remaining 13 loci were used for further analysis.

Data processing

Null allele frequencies for the microsatellite loci were examined using MICRO-CHECKER 2.2.3 (van Oosterhout et al. 2004) and CERVUS 3.0.6 (Kalinowski et al. 2007). Allelic frequencies, expected and observed heterozygosities were estimated using ARLEQUIN 3.5.1.2 (Excoffier et al. 2005). The same software was also used to test for Hardy Weinberg equilibrium (HWE) and linkage equilibrium. Bonferroni corrections were used for both calculations. Allelic richness was examined in FSTAT 2.9.3.2 (Goudet 1995). Statistical significance was examined using unpaired t tests.

Maternity, paternity and parent pairs with known sexes were determined using the likelihood-based approach of CERVUS. Loci with null allele frequencies greater than 0.05 were excluded to avoid false parental assignments. Two levels of confidence were set at 80% (relaxed) and 95% (strict). Positive LOD scores (i.e. the logarithms of the likelihood ratios) were compared to identify the most likely parents for each offspring. In 2012 and 2013, five of the six females (12654, 12656–12659) and three of the 14 males (12660, 12661, 12662) for which samples were available were kept together for breeding. Thus, using five candidate mothers and three candidate fathers, simulations of 10,000 offspring genotypes were run, each at a sampling rate of 100%. The proportion of mistyped loci was set to

0.01. Alternatively, six candidate mothers and 14 candidate fathers, with the remaining settings unchanged, were used because insemination prior to the separation of the breeding group could have occurred. In addition, relationships among terrapins were inferred using ML-RELATE (Kalinowski et al. 2006). In contrast to CERVUS, this Maximum Likelihood-based software also estimates full sibling and half sibling relationships as well as parent-offspring (PO) relationships. Moreover, there is a null allele correction implemented in ML-RELATE, based on a Monte Carlo randomization test (Guo & Thompson 1992) and a U test (Rousset & Raymond 1995). Thus, estimates with and without null allele correction were compared using a 95% confidence level and 10,000 randomizations. Specific hypothesis tests were performed using 100,000 simulations. To assess whether a PO relationship is statistically more likely than a full sib or half sib relationship, the specific hypothesis test of ML-RELATE was run. Networks for relationships of individual terrapins were visualized using PAJEK 3.14 (Batagelj & Mrvar 2003).

Results

Even though the captive-bred terrapins showed generally less genetic diversity (average allelic richness of 4.97) compared to the wild-caught ones (average allelic richness of 6.55; Table 1), the differences were statistically not significant ($t = 1.09$, $p = 0.29$). Also, the number of alleles per locus was higher in wild-caught terrapins, with their private alleles corresponding to those whose alleles were not transmitted to the offspring (Tables 1 and S8). In the F_1 generation, 73.9% of alleles per locus were preserved. Differences between expected and observed heterozygosities within each group were not significant (wild: $t = -0.026$, $p = 0.979$; captive: $t = 0.514$, $p = 0.612$). Furthermore, the data also showed no significant difference with respect to observed and expected heterozygosities between the groups (H_O: $t = -0.117$, $p = 0.907$; H_E: $t = 0.402$, $p = 0.691$). The deviation from HWE in the F_1 generation for some loci (Table 1) is expected because the animals originate from a few breeding pairs.

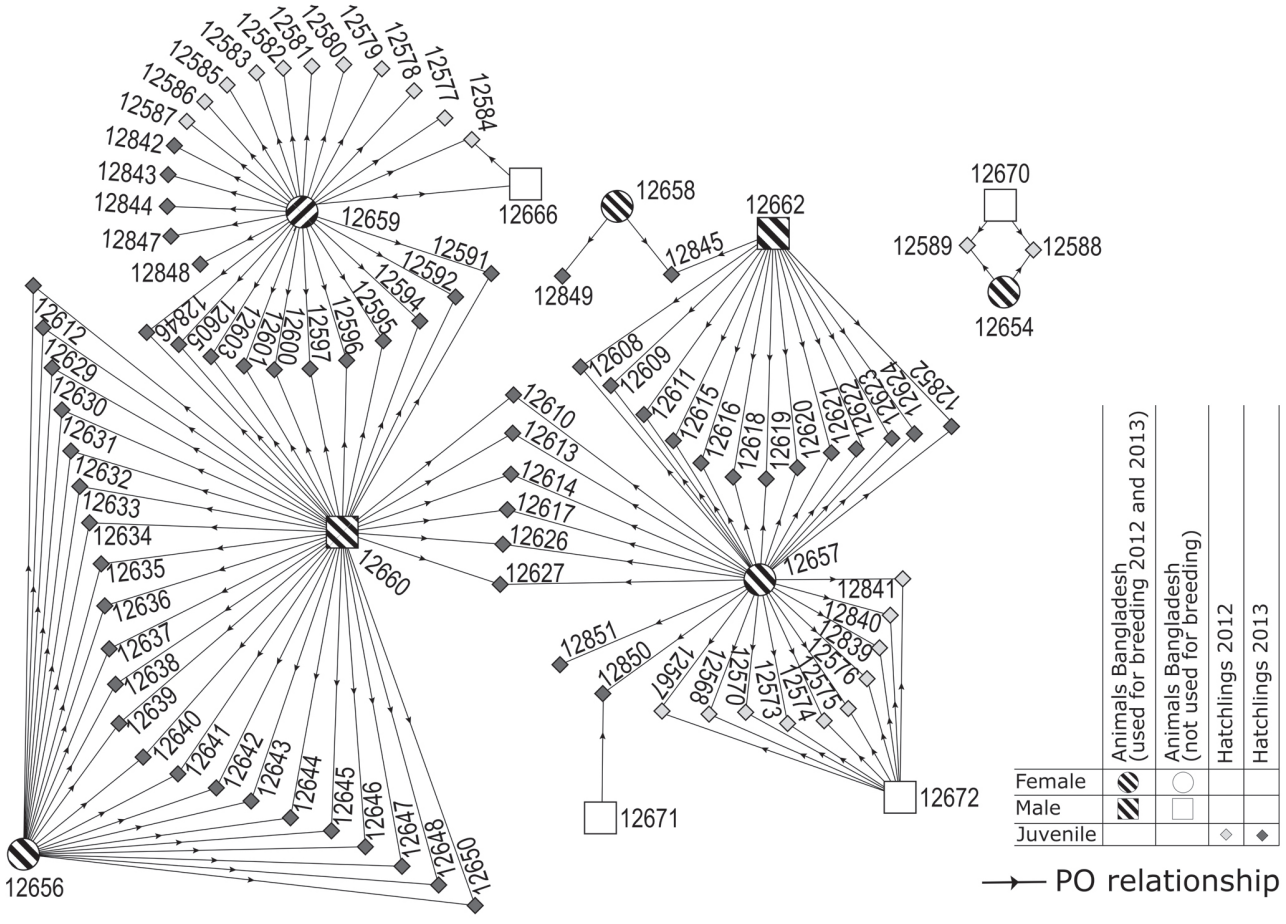

Figure 2. Relationship of adult terrapins and hatchlings in Bangladesh, derived from CERVUS and ML-RELATE calculations. Females are represented by circles, males by squares, and juveniles by diamonds. Numbers are individual lab codes. Hatchlings from 2012 are marked in light grey, hatchlings from 2013 in dark grey. Black lines show PO relations, arrows point from parent towards the offspring. Two-headed arrows indicate PO relations between adults with unknown age. For further explanation, see inset.

The results of ML-RELATE generally agreed well with the assigned parent-offspring relations (PO) revealed by CERVUS. Also the assigned full sib (FS) and half sib (HS) relationships supported the results of CERVUS.

Juveniles from four nests hatched both in 2012 and 2013. The hatchlings were expected to originate from the five females (12654, 12656–12659) and three males (12660, 12661, 12662) that were then kept together. For each hatchling, the parents were identified using CERVUS and ML-RELATE. In 2012, all hatchlings from the same nest had the same mother. Accordingly, female 12657 was the mother of clutch 1 (hatchling 12567) and clutch 2 (hatchlings 12568, 12570, 12573–12576, 12839–12841). For both clutches, a male was revealed as the father (12672) that was not kept with the females in 2012. Clutch 3 (hatchlings 12577–12587) was laid by female 12659. This clutch was sired by two fathers, but only for hatchling 12584 the father could be identified (male 12666, again not kept with the females in 2012). The father of the remaining hatchlings remains unknown. Clutch 4 (hatchlings 12588, 12589) originated from female 12654 and from another male (12670) not kept with the females in 2012 (Fig. 2; Supplementary Table S5).

For the 2013 season, female 12659 was identified as mother of the 16 hatchlings from clutch 1 (12591, 12592, 12594–12597, 12600, 12601, 12603, 12605, 12842–12844, 12846–12848) and male 12660 as father except for juveniles 12842–12844, 12847 and 12848, whose father was another unidentified terrapin. Hatchlings from clutch 2 (12845, 12849) resulted from female 12658. The father of hatchling 12845 was male 12662, while the father of hatchling 12849 was again an unsampled male. Female 12657 was the mother of the 20 hatchlings from clutch 3 (12608–12611, 12613–12624, 12626, 12627, 12850, 12851). However, one hatchling (12612) from this clutch was assigned to another mother (female 12656), which was also revealed as mother of clutch 4 (hatchlings 12629–12648, 12650, 12852). In the latter clutch, one juvenile (12852) was identified as offspring of female 12657 and male 12662. The fathers of the hatchlings from nest 3 are males 12660, 12662 and 12671, while all hatchlings from nest 4 most likely originate from male 12660 (Fig. 2; Supplementary Table S6). As is obvious, many hatchlings have unidentified fathers or fathers (12666, 12670–12672) that were evidently not kept together with their mothers in 2012 and 2013. Furthermore, one egg each of clutches 3 and 4 of 2013 seem to have been inadvertently switched during incubation (Supplementary Table S6).

The specific hypothesis test of ML-RELATE suggests that most wild-caught terrapins are related in a degree of half siblings. Two PO relationships as well as one full sibling relationship were detected among the adult terrapins (Fig. 3; Supplementary Table S7).

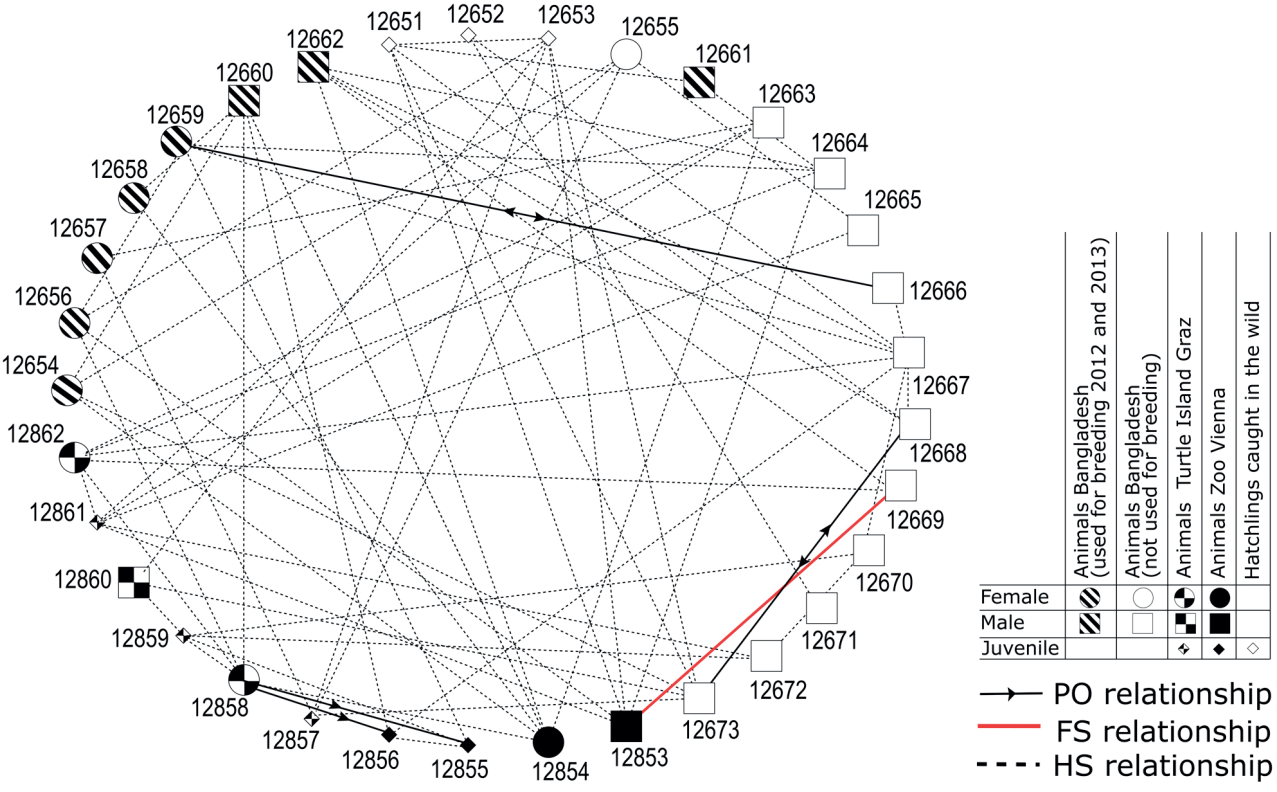

Figure 3. Relationship of wild-caught terrapins as well as of animals bred in Vienna Zoo and Turtle Island, derived from CERVUS and ML-RELATE calculations. Numbers are individual lab codes. The red line shows full sib relations; dashed lines, half sib, grandparent-grandchild, uncle/aunt-nice/nephew, or first cousin relations. For further explanation, see Figure 2 and inset.

Table 2. Recommended mating combinations for captive adult *Batagur baska* (parent-offspring and half sib relationships excluded; the only full sib relationship found refers to two males). * – combinations that produced offspring in 2012 and 2013.

♀ \ ♂	12660	12661	12662	12663	12664	12665	12666	12667	12668	12669	12670	12671	12672	12673	12853 (Vienna)	12860 (Graz)
12654	**no**	yes	yes	yes	yes	yes	yes	yes	yes	yes	**no***	yes	yes	**no***	yes	yes
12655	yes	yes	yes	yes	yes	**no**	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
12656	**no***	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	**no**	yes
12657	**no***	yes	**no***	**no**	yes	yes	yes	yes	yes	yes	yes	yes	**no***	yes	yes	yes
12658	**no**	yes	**no***	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
12659	**no***	yes	yes	yes	**no**	yes	**no**	**no**	yes	yes	yes	yes	yes	yes	yes	yes
12854 (Vienna)	**no**	yes	**no**	**no**	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
12858 (Graz)	**no**	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
12862 (Graz)	yes	yes	yes	**no**	**no**	yes	yes	**no**	yes	**no**	yes	yes	yes	yes	yes	yes

Discussion and conclusions

Our study is one of the relatively few examples for the application of conservation genetics in a captive breeding programme for reptiles (see the review in Witzenberger & Hochkirch 2011). These authors surveyed 188 studies using conservation genetic approaches for breeding programmes. The majority of studies applied microsatellites, with a clear focus on mammals and birds, and only 9% were referring to reptiles. However, turtles belong to the most endangered of any of the major groups of vertebrates and their status is paralleled only by the similarly endangered primates (Rhodin et al. 2017). Among turtles, the northern river terrapin (*Batagur baska*) is one of the most critically endangered species of the world (Rhodin et al. 2011). For the conservation of this ecologically extinct species, and many other turtle species, captive breeding plays a key role (Williams & Osentoski 2007, Weissenbacher et al. 2015). Yet, most captive breeding projects are not monitored genetically and only recently attention has been paid to the pedigree or relatedness of breeders using conservation genetic approaches (Çilingir et al. 2017 for the closely related *B. trivittata*), even though Williams & Osentoski (2007) recommended the implementation of such measures one decade ago.

The breeding projects for *B. baska* in Bangladesh and Austria aim to preserve as much as possible the current genetic diversity. To achieve this goal, we developed a powerful microsatellite marker system to assess individual relationships. This marker system allows estimating the relationship of wild-caught and captive-bred individuals, and thus constructing pedigrees to plan future breeding.

In Bangladesh, 84 juveniles hatched during the seasons 2012 and 2013. We studied these terrapins, the wild-caught breeders, and all terrapins from the Austrian breeding colonies. Our results are promising. The first captive-bred generation retained approximately 75% of the genetic diversity of the wild-caught founder individuals (Table 1), even though no breeding strategy had been implemented yet. We are confident that with introducing a breeding strategy combining less related or unrelated individuals, the percentage of preserved genetic diversity can be significantly increased.

According to our results, most wild-caught terrapins are related (Fig. 3). Given the long life expectancy of turtles, this situation suggests that the wild population experienced a severe decline long ago. The few survivors are largely related at the level of half sibs (or first cousins, aunts/uncles-nices/nephews or grandparents-grandchildren). For conservation purposes, the present genetic diversity should be preserved to the greatest extent possible. Thus, the reproduction of closely related terrapins has to be avoided. Based on our data, we suggest that selective breeding should be implemented, and some mating combinations be avoided (Table 2). Moreover, we recommend that in following seasons the males 12661, 12663–12665, 12667–12669, and 12673, which did not contribute to previous offspring, should be used for breeding. In addition, the adults from the two breeding colonies in Austria should also be involved for breeding in Bangladesh. The highest priorities have those unrelated individuals listed in Table 2 that have not bred before.

A remarkable finding for the colony in Bangladesh was that some captive males contributed to offspring that must have mated with the females prior to the reproductive seasons of the present study. Moreover, we could not identify the fathers of some juveniles (Fig. 2). Even if the latter should be all offspring from the same unsampled male that died in 2013 in Bangladesh, it was not kept together with the females in 2012 and 2013. This provides unambiguous evidence for sperm storage in *B. baska*. Also females of other turtle species are known to store sperm in their oviducts, so that clutches can be fertilized long after the last mating (Gist & Jones 1989, Pearse & Avise 2001, Pearse

et al. 2001, Roques et al. 2004, 2006, Johnston & Rand 2006). Our results also provide evidence for multiple paternity in *B. baska* (Fig. 2; Tables S5 and S6), a phenomenon supported by sperm storage and thought to increase fertility rate (Olsson et al. 1996) and genetic diversity (Andersson 1994).

Our microsatellite marker system developed for *B. baska* also promises to be informative for the five other species of the genus *Batagur*. All species are considered by the last IUCN Red List assessment as either Critically Endangered (*B. affinis*, as part of *B. baska*; *B. borneensis*; *B. kachuga*; *B. trivittata*) or Endangered (*B. dhongoka*) (Rhodin et al. 2017), and for many taxa captive breeding projects are underway. Microsatellite primers usually perform well within, and often beyond, the same turtle genus (unpubl. observ.; see also Schwarz et al. 2003, King & Julian 2004, Vamberger et al. 2011, 2017, Tiedemann et al. 2014). Thus, we expect to have provided a most useful tool for preserving genetic diversity in breeding projects for *Batagur* species in general.

Compared to the ddRAD-Seq approach to generate SNPs used by Çilingir et al. (2017) for *B. trivittata*, our microsatellites are an easy and affordable method to generate an informative marker set that does not need high-quality DNA. Our approach can be easily repeated by any basic molecular genetic laboratory.

Finally, we explicitly recommend implementing conservation genetic assessments for other captive breeding projects of turtles to preserve a maximum of genetic diversity and to avoid inbreeding depression.

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Supplementary material

Supplementary Table S1. Studied *Batagur baska*.

Supplementary Table S2. Studied captive-bred terrapins from the Project Batagur, Bangladesh.

Supplementary Table S3. Microsatellite loci tested for cross amplification in *Batagur baska*, multiplex sets, allele size ranges and number of alleles of the individual loci.

Supplementary Table S4. Thermocycling conditions for multiplex PCRs for microsatellites.

Supplementary Table S5. Offspring from 2012 nesting season.

Supplementary Table S6. Offspring from 2013 nesting season.

Supplementary Table S7. Assigned relationships among wild-caught terrapins.

Supplementary Table S8. Private alleles per locus of individual wild-caught terrapins.