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Short communication

Multiple paternity and sperm storage in captive Hermann's tortoises, *Testudo hermanni boettgeri* determined from amniotic fluid adhering to the eggshell

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ABSTRACT

We identified multiple paternity in 52.9% of the clutches of Hermann's tortoise *Testudo hermanni boettgeri* using polymorphic microsatellite markers. In addition we demonstrated sperm storage across seasons. DNA was extracted from the amniotic fluid adhering to the eggshell's inner surface, a procedure suitable for easy, non-invasive DNA sampling in conservation and breeding programs. To improve the informative value of monomorphic single tandem repeat (STR) markers we additionally analyzed single nucleotide polymorphism (SNP) variability.

Promiscuous mating is widespread especially in vertebrates without strong social bonds, but extra-pair mating also occurs in so-called monogamous species (e.g. sharks [e.g. Refs. [1,2]], reptiles [3,4], tits [e.g. Refs. [5,6]], hedgehogs [7], man [8]). The females of polyandrous species are equipped with oviductal tubules [9] where viable sperm can be stored at least over short periods of time. As a consequence, siblings within one clutch can be sired by different males. Among chelonians, who do not form complex social structures nor do they provide parental care, multiple paternity was found in all marine turtle species except the leatherback turtle [10,11], and in all freshwater turtles tested [12–20]. In tortoises, multiple paternity occurs in the desert tortoise (*Gopherus agassizii* [21,22]), the Mediterranean spur-thighed tortoise (*Testudo graeca* [23]), the central Asian tortoise (*Testudo horsfieldii* [24]), and Hermann's tortoise (*Testudo hermanni hermanni* [25]).

The parentage of 83 *T. hermanni boettgeri* from two private captive breeding colonies (group F and H, 8 adult females, 10 adult males) was analyzed during the breeding seasons 2010/2011 (see supplementary information S1, supplementary Table ST1) using a group of specific polymorphic microsatellites developed for chelonians.

We successfully isolated DNA from amniotic fluid adhering to the inner surface of the eggshell of 62 live hatchlings and from 5 animals that had died during incubation thereby avoiding the difficult and potentially stressful sampling of saliva or blood in very young animals. This approach was first used in birds and alligators [26,27].

In adult potential parents (n = 18; H01–H04, H7–H10, F01–F04, F07, F72, F101, F135, F201, F201a) and semi-adult animals (n = 6; F102, F103, F104, F119, F120, F125) from earlier clutches, DNA was isolated from their blood, sampled from the dorsal coccygeal or femoral vein, and dried on filter paper. In addition, tissue samples of frozen and ethanol fixed animals from earlier clutches (n = 10; F108, F109, F117, F118, F123, F126, F128, F129, F131, F132) were collected, and DNA was isolated. All samples were stored at −20 °C until analysis.

DNA was isolated and purified using the tissue or blood protocol variant of the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Twelve gene loci, that were characterized by their highly

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polymorphic length variation, were amplified using polymerase chain reaction (PCR). Amplification products were resolved on an ABI3730xl, and sized and scored with the GeneMapper® software. Statistical analysis was performed with CERVUS 3.0 ([28]; supplementary information S4, supplementary Tables ST5, ST6). The following polymorphic microsatellite markers developed for tortoises were used in our genotyping experiments: Test10, Test71, Test88 [29], TW161, TWR106 [30], RAD284 [31], and SK60 that was developed for *T. hermanni* and *Testudo kleinmanni* in our laboratory (supplementary information S2, supplementary Table ST2).

Multiplex PCR was performed under the following conditions: initial denaturation at 94 °C for 10 min, followed by 30 cycles at 94 °C for 1 min, at 58 °C for 1 min, at 72 °C for 1 min and at 72 °C for 45 min, and a break at 15 °C. The final reaction volume was 20 μl: 2 μl DNA, 2.5 μl $MgCl_2$ (25 mM), 0.2 μl Promega GoTaq (5u/μl), 3.2 μl dNTP mix (1.25 mM), 1 μl forward and 1 μl reverse primer (10 μM) for each marker, 4 μl Colorless GoTaq Flexi Buffer (Qiagen), filled with distilled water to 20 μl. We applied 3 multiplex reactions: MP1: Test10, Test71, TW161; MP2: Test88, TWR 106, RAD 284; MP 3: SK 60. Although the annealing temperature used in the multiplex reaction is a compromise and may not be optimal for the individual PCR amplification reactions, PCR products were of different sizes and could be robustly amplified. One exception was marker Test88, which was replaced by marker TW161 in further analyzes (supplementary Tables ST5, ST6). With this methodological approach we were able to analyze multiple microsatellite markers despite the limited quantity of DNA.

In order to obtain additional information from monomorphic markers, PCR products were amplified and analyzed by direct DNA sequencing in three parent turtles and eight of their offspring (supplementary information S3, supplementary Table ST3, ST4) that were homozygous for one or several markers. This analysis should reveal potential single nucleotide polymorphism (SNP) within the STR-repeat or between the STR-repeat and the primer loci that could in turn provide additional information about paternity, if polymorphic. Of course, these analyses are only valuable for markers where both alleles can be amplified and are not subject of allele drop-out.

Employing seven informative microsatellite markers (Test10, Test71, Test88, TWR106, RAD284, SK60, TW161) we could unequivocally determine the fathers of all juveniles at an 80% or 95% confidence level. The mothers' identity determined by DNA analysis, unequivocally confirmed our observations of the animals during oviposition. In some cases, several markers were monomorphic, e.g. only one allele could be identified. This can be caused by allele drop out, but can also be due to a problem of the STR-analysis, which is sometimes encountered in exotic species or wild animals where markers are used that were originally developed for another subspecies or for a related species. In these cases the markers are often not as informative as for the original species. Furthermore, when working with degraded material it is not always clear whether the marker is really homozygous or whether a second allele cannot be amplified. Thus, for two markers (TW161 and Test71), we analyzed the flanking regions of the microsatellite for polymorphic SNP. This additional genetic variability potentially increases the informative value of the locus. As an example we show the sequencing results of the TW161 locus for two families ((H01/H07, parents; H69, H71, H73, H76, juveniles); (H01/H10, parents; H70, H72, H74, H68, juveniles)) (supplementary information S3, supplementary Table ST3, ST4). In individuals with previously identified monomorphic microsatellite markers, we could identify two polymorphic SNPs confirming the corresponding STR-based parentage.

Multiple paternity was found in two of six clutches from colony F, and in seven of nine clutches from colony H. The offspring in eight of these clutches was fathered by two different males, the offspring of the remaining clutch was derived from three different males. Thus, in 52.9% of the clutches analyzed offspring was fathered by more than one male.

We identified three cases of sperm storage (F121, F218–F221, H28) where the fathers determined by DNA analysis were not part of the breeding groups during the season we analyzed indicating that mating had occurred during the previous season and that sperm had been stored.

In the wild, tortoises are promiscuous [3,32,33]. Generally, multiple mating can have various advantages for the female ranging from decreasing harassment by aggressive males ("convenience polyandry" [2]) to direct benefits such as nuptial gifts and help during raising of the young [3,4]. In chelonians, however, indirect benefits such as "trading up" the quality of the sperm, promoting sperm competition, cryptic female choice, and "bet-hedging" i.e. the production of diverse geno- and phenotypes within clutches to be better equipped for environmental changes [4] seem to be more likely but these factors are mostly untested. All these benefits are invariably linked to the ability to store sperm, either within a season, e.g. mating in spring and inseminating consecutive clutches during the summer, or across seasons when sperm is stored for up to 3–4 years [3,4,34]. The mating and courtship behavior of *T. hermanni* has been described in detail [e.g. Refs. [25,32–38]]. In natural habitats, population density of *T. hermanni* can be rather low (from <3 individuals/ha [39], to ~40 individuals/ha [35,36,40]), and mating occurs during the rare encounters. Olfactory, visual, tactile, and auditory signals are important for species and mate recognition and provide honest cues for mate quality [37,41]. Thus they represent important signals for female strategies as cryptic female choice or bet-hedging [4]. Our animals produced 2-3 clutches per season, which is similar to the situation found in the wild [25,32,42]. In both colonies, the ability for sperm storage was confirmed. In colony F, multiple paternity was shown in 33%, in colony H in 78% of the clutches. Fertilization success was 85% in colony F and 95% in colony H, and thus much higher than the <60% described for permanently co-housed *T. hermanni* by Jasser-Häger and Winter [43]. Our sample is too small to deduce influences of the breeding scheme on the prevalence of multiple paternity or fertilization success.

Taken together, multiple paternity in *T. hermanni boettgeri* was identified in 52.9% of the clutches analyzed. This rate is higher than described for *T. graeca* (20% [23]) and *T. horsfieldii* (27% [24]), and comparable to *Gopherus* (50–57% [21,22]) kept in captivity, or in a semi-natural environment. In a planned mating study Cutuli et al. [25] found a slightly lower rate of multiple paternity in a captive colony of *T. hermanni hermanni*. Multiple paternity ranges from 10% in *Emys orbicularis* [16] and some populations of *Podocnemis expansa* [15] up to 50–100% in *Chelonia midas* [44,45], *Podocnemis expansa* [13], and *Podocnemis unifilis* [17] with most rates lying between 20% and 80% (*Lepidochelys olivacea* 20% [46], *Caretta* 31–33% [47,48], *Chrysemys picta* 33% [14], *Lepidochelys kempi* 58% [49], *Emydoidea blandingii* 60–80% [18], *Chelydra serpentina* 66% [12], *Podocnemis erythrocephala* 80% [19], *Elseya albagula* 83% [20]). Thus, the incidence of multiple paternity in Hermann's tortoise seems similar to other tortoises and turtles even though direct comparisons are difficult due to different sample sizes and living conditions.

In conclusion, the data described here extend our knowledge about multiple paternity and sperm storage to a further tortoise taxon, *T. hermanni boettgeri* indicating that these traits are widespread in chelonians in general. To the best of our knowledge, this is the first study in chelonians to employ the non-invasive method of collecting DNA samples from amniotic fluid adhering to the inner surface of the eggshell. The supplementary SNP analysis proved to