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Research article

The necessity of genetic screening for proper management of captive crocodile populations based on the examples of *Crocodylus suchus* and *C. mindorensis*

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Abstract
Based on sequences of mitochondrial and nuclear genes, we report on a screening of 11 presumed Nile crocodiles from various European zoos, of which five (from four facilities) turned out to be western Nile crocodiles, *Crocodylus suchus*, the recently resurrected name applied to the western genetic lineage of *Crocodylus niloticus sensu lato*. We also provide evidence for a pure species-level genetic background of six additional *Crocodylus mindorensis* from a European zoo facility, a species that is known to hybridise with *Crocodylus porosus*. Our results are based on a limited number of genetic markers and thus might miss backcrossed hybrid specimens, but they provide an important basis for the establishment of conservation breeding programmes, already in place for *C. mindorensis* and contemplated for *C. suchus*. We found evidence for possible genetic admixture between *C. suchus* and *C. niloticus* in a specimen found in Lebanon, possibly representing a released captive-bred hybrid. We reiterate the need for such basic genetic screening especially in morphologically cryptic and poorly studied species in the context of ex-situ conservation breeding, to avoid erroneous species identification and overlooking of unknown evolutionary lineages.

Introduction

As a basis for a conservation breeding programme for the threatened Philippine crocodile (*Crocodylus mindorensis*) in Europe, Hauswaldt et al. (2013) conducted a first genetic screening of individuals kept in European zoo facilities. This research was imperative, because hybrid individuals, derived from crosses of the Philippine crocodile with the Saltwater crocodile (*C. porosus*), were identified at a captive breeding facility in the Philippines (Tabora et al. 2012; Hinlo et al. 2014). Given the potential negative effects of interspecific hybridisation (Rhymer and Simberloff 1996, Allendorf et al. 2001), such hybrids should be excluded from ex-situ conservation breeding to conserve unpolluted species-specific gene pools.

By using mitochondrial DNA (mtDNA) and nuclear DNA (nucDNA) markers, Hauswaldt et al. (2013) provided evidence that the genetically screened *C. mindorensis*, all originating from captive sources in the Philippines and thereafter imported to Europe, were pure *C. mindorensis*. However, one presumed pure *C. mindorensis* previously held in zoos in Wroclaw (Poland) and Dvur Kralove (Czech Republic) and thereafter kept at Zurich Zoo, turned out to be a western Nile crocodile (*C. suchus*), the western lineage of *C. niloticus sensu lato*, which was only recently resurrected as a distinct taxon (Schmitz et al. 2003; Hekkala et al. 2011). Because this was the first published record of a *C. suchus* kept in a European facility, T. Ziegler appealed to members of the European Association of Zoos and Aquaria at the Amphibian and Reptile Taxon Advisory Group conference held in Rome in April 2012, to provide blood or tissue samples of Nile crocodiles for genetic screening. This would permit the identification of other *C. suchus* that may have been misidentified as *C. niloticus sensu stricto*, and thereby prevent accidental captive hybridisation between these two distinct taxa. The genetic data would also provide the setting for a breeding programme for the rare and poorly known *C. suchus*.

Table 1. Crocodile specimens newly sequenced in the present study. Mitochondrial DNA (mtDNA) assignment is given according to the clade to which a sample grouped in the phylogenetic analyses (Cm, *C. mindorensis;* Cn, *C. niloticus*; Cs, *C. suchus*). The DLOOP fragment was sequenced in all samples, some samples were also sequenced for 12S. Haplotypes of the nuclear LDH-A gene fragment are according to Hauswaldt et al. (2013). Haplotypes I and II are typical for *C. mindorensis*, IV for *C. suchus* and VII for *C. niloticus.* Note that LDH-A sequences obtained for some of the new specimens are slightly shorter than those of the previous study. Therefore it is possible that they might represent other (previously unrecorded) haplotypes (however, this would not influence species identification).

Inferred taxon	Specimen	Number	Facility	mtDNA	LDH-A
C. niloticus	Irina	968000004606342	Halle Zoo, Germany	Cn clade (DLOOP)	Haplotype VII
C. niloticus	Ginalu	968000004614141	Halle Zoo, Germany	Cn clade (12S, DLOOP)	Haplotype VII
C. niloticus	NEST	968000004656000	Halle Zoo, Germany	Cn clade (DLOOP)	Haplotype VII
C. niloticus	1874		Crocodiles of the World, UK	Cn clade (DLOOP)	Haplotype VII
C. niloticus	RD177		Crocodiles of the World, UK	Cn clade (DLOOP)	Haplotype VII
C. niloticus/suchus	Lebanon, wild-caught		Crocodiles of the World, UK	Cn clade (DLOOP)	Haplotype IV
C. suchus	Mexin, female	968000004606437	Halle Zoo, Germany	Cs clade (12S, DLOOP)	Haplotype IV
C. suchus	Female		Copenhagen Zoo, DK	Cs clade (12S, DLOOP)	Haplotype IV
C. suchus	Male		Copenhagen Zoo, DK	Cs clade (12S, DLOOP)	Haplotype IV
C. suchus	unnamed		La Ferme aux Crocodiles, France	Cs clade (12S, DLOOP)	Haplotype IV
C. suchus	L00088, female	985101020063929	Zoo Lyon, France	Cs clade (12S, DLOOP)	Haplotype IV
C. mindorensis	Golda	985120027838974	Protivin Crocodile Zoo, CZ	Cm clade (12S, DLOOP)	Haplotype II
C. mindorensis	Karel	956000002283302	Protivin Crocodile Zoo, CZ	Cm clade (12S, DLOOP)	Haplotype II
C. mindorensis	Jack	956000002339585	Protivin Crocodile Zoo, CZ	Cm clade (12S, DLOOP)	Haplotype II
C. mindorensis	Minda	985120029043711	Protivin Crocodile Zoo, CZ	Cm clade (12S, DLOOP)	Haplotype II
C. mindorensis	Monty	985120029025105	Protivin Crocodile Zoo, CZ	Cm clade (12S, DLOOP)	Haplotype II
C. mindorensis	Světlana	985120024073321	Protivin Crocodile Zoo, CZ	Cm clade (12S, DLOOP)	Haplotype II

In this paper we present the results of our first round of genetic screening of Nile crocodiles held in European zoos for *C. suchus*. We also report on screening results of additional *C. mindorensis* held in Europe, which had not been available at the time of our previous study (Hauswaldt et al. 2013), to add these individuals to the conservation breeding programme for Philippine crocodiles. Furthermore, we correct minor graphical mistakes in Figure 1 of Hauswaldt et al. (2013).

Methods

In addition to the crocodile specimens studied by Hauswaldt et al. (2013), 17 crocodiles were sequenced from European zoos: six presumed Philippine crocodiles from Protivin Crocodile Zoo and 11 Nile crocodiles *sensu lato* from Copenhagen Zoo (n = 2), Crocodiles of the World (n = 3), Halle Zoo (n = 4), La Ferme aux Crocodiles (n = 1), and Lyon Zoo (n = 1) (Table 1). DNA was extracted from a variety of samples, including blood, saliva swabs and shed skin, mostly preserved in pure ethanol. We sequenced fragments of two mitochondrial genes (12S rRNA and DLOOP) and the nuclear LDH-A gene, as well as the c-myc gene in presumed *C. mindorensis*, following protocols in Hauswaldt et al. (2013). Newly determined sequences were submitted to Genbank (accession numbers KM881486-KM881514). All specimens were sequenced for DLOOP but only a subset for 12S. Because mitochondrial genes are linked, they reflect the same evolutionary history. Both DLOOP and 12S show quite a large variability and allow easy assignment to species specific clades. Therefore, we discuss the mitochondrial data not in terms of haplotypes (as in Hauswaldt et al. 2013), but instead simply state to which clades the new haplotypes were assigned according to our phylogenetic analysis.

All sequences were checked in CodonCode Aligner (CodonCode Corp.) and further analysed in MEGA 5 (Tamura et al. 2011). To show mtDNA relationships, we calculated a Maximum Likelihood tree (Fig. 1) with MEGA, with a general-time reversible substitution model with gamma-shaped distribution and invariant sites (GTR+I+G). Node support was estimated by 500 bootstrap replicates. Note that the purpose of this single-gene tree is not to infer phylogenetic relationships among crocodile species but to show clustering of individuals to species specific lineages.

Results

Crocodylus niloticus/suchus

The focal group of *C. niloticus/C. suchus* specimens could be assigned to two clearly delimited subclades based on the mtDNA gene fragments. The *C. suchus* subclade (Fig. 2) contained one specimen from Halle Zoo (Germany), a crocodile pair from

Copenhagen Zoo (Denmark), and two specimens from two facilities in France (Lyon Zoo and La Ferme aux Crocodiles). Our data therefore indicate the presence of multiple captive individuals of this species in European zoos. All of these specimens were also characterised by LDH-A haplotype IV (numbering according to Hauswaldt et al. 2013), which is typical for *C. suchus* (and possibly *C. siamensis*) according to the limited data available to date. No heterozygous nucleotides were detected in the newly determined LDH-A sequences.

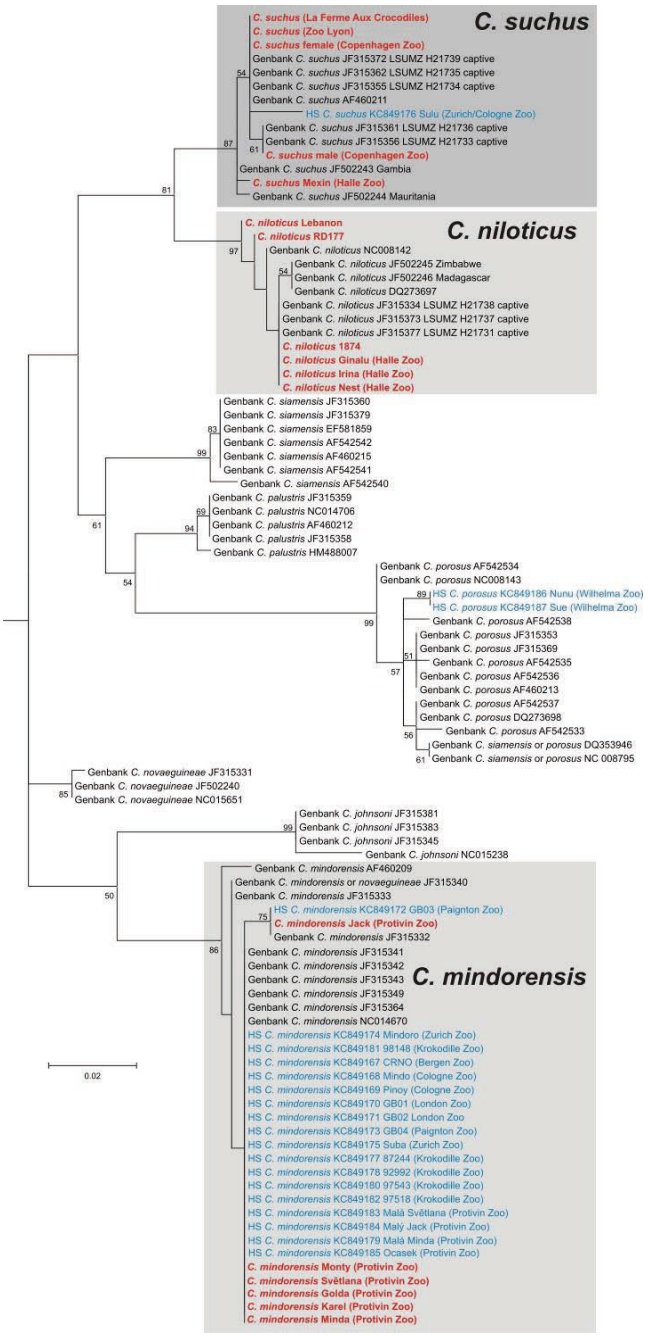

Figure 1. Maximum Likelihood tree based on 623 bp of the mitochondrial control region (D-Loop). Sequences marked with red are from this study, those marked in blue and prefixed with HS are from the previous study (Hauswaldt et al. 2013) with the respective Genbank number and all other sequences were retrieved from Genbank. *Osteolaemus tetraspis* was used as an outgroup. Numbers on the branches are support values in percent from a bootstrap analysis (not shown if <50%). The purpose of the tree is not to accurately reconstruct the phylogeny of *Crocodylus* but to summarise the clustering of individuals into species-level lineages.

The specimen sequenced from Lyon Zoo was a small female, hatched 14 July 2000, which derived from a breeding pair that arrived in Lyon in 1976 from a French tannery school (Guillaume Douay, pers. comm.). As both mtDNA and LDH-A unanimously assigned this offspring to *C. suchus,* without indication of heterozygosity in LDH-A, it can be concluded that the parental pair (still kept in Lyon Zoo) are also *C. suchus* instead of *C. niloticus*. The *C. suchus* held in La Ferme aux Crocodiles is presumed to originate from Ivory Coast (Samuel Martin, pers. comm.).

Three specimens from Halle Zoo, as well as two specimens from facilities in the UK, were assigned by mtDNA to *C. niloticus* ("eastern *C. niloticus*"), and had LDH-A haplotype VII, which is typical for this species.

One individual from Lebanon included in this study (Fig. 3) is of particular interest, because mtDNA assigned it unambiguously to *C. niloticus* while its LDH-A haplotype IV is typical for *C. suchus.* This suggests either a case of haplotype sharing among the two species in this nuclear gene, or the possibility of hybridisation. Either way, this individual from Lebanon most probably represents a suspended animal that was subsequently caught in the wild, and not deriving from a native population (Shaun Foggett, pers. comm.).

Crocodylus mindorensis

We have now been able to screen all ten crocodiles that Protivin Crocodile Zoo (Czech Republic) had received from the Avilon Zoo in the Philippines, while only four samples were available for our previous study (Hauswaldt et al. 2013; three unnamed juveniles

Figure 2. *Crocodylus suchus* held at Cologne Zoo (above) and Halle Zoo (below). Photos: Anna Rauhaus (above), Hans-Günter Hofmann (below).

Figure 3. The Lebanon wild-caught individual held at Crocodiles of the World (above), of which mtDNA assigned it unambiguously to *C. niloticus* while its LDH-A haplotype IV is typical for *C. suchus*, and Jack (*Crocodylus mindorensis*) held a Protivin Crocodile Zoo (below). Photos: Shaun Foggett (above), Miroslav Prochazka (below).

and the specimen named Ocasek). The three unnamed juveniles in Hauswaldt et al. (2013) correspond to the specimens named Malá Světlana (956000002289357), Malá Minda (956000002275518), and Malý Jack (956000002314019). According to our screening data, the individuals named Golda, Jack, Karel, Minda, Monty, and Svetlana could also all be assigned to the mitochondrial clade of *C. mindorensis*, and all were homozygous for haplotype II in LDH, which is typical for this species (and otherwise only observed in *C. novaeguineae*). To exclude the possibility that some of the specimens were actually *C. mindorensis/novaeguineae* hybrids, we also sequenced a fragment of the c-myc gene for which these species have different haplotypes (while they are known to share LDH-A haplotypes). All newly sequenced specimens were homozygous for haplotype I, typical for *C. mindorensis* (not shown in Table 1), and we therefore identified all individuals as pure *C. mindorensis*.

In order to correctly place our results in context, we herewith would like to provide some corrections to Figure 1 in Hauswaldt et al. (2013), as we realised that two of the haplotype networks contained a number of mistakes (although they did not affect the conclusions of the paper). In the c-myc network, haplotype V is individual #124, VII is individual #125, IX is individual #120, and XI is individual #119. Furthermore in the D-loop network, (i) haplotype 4 should have been coloured blue, (ii) haplotype 17 refers to individual #85 instead of #80, (iii) haplotype 19 refers to individual #126 (AF542541) instead of #35, (iv) only one (not two) mutational steps between haplotypes 12 and 13, (v) only four (not five) steps on the branch connecting haplotype 30 and the adjacent note, (vi) one extra step needed on the branch connecting haplotype 27. Revised haplotype networks are available upon request.

Discussion

Until now, the species identity of most Nile crocodiles in European zoos and breeding facilities has remained elusive because no reliable diagnostic characters are known to distinguish between *C. niloticus* and *C. suchus.* Only one positively identified *Crocodylus suchus* existed in a European zoo facility, and this specimen had been coincidentally identified during a study on captive *C. mindorensis* (Hauswaldt et al. 2013); this animal is now housed at the Cologne Zoo. In the present study, we identified additional individuals of *C. suchus* at four other European captive facilities. As these are adults of different sexes, it will now be important to establish a breeding programme for *C. suchus*. The breeding pair at Copenhagen Zoo has already reproduced successfully (Flemming Nielsen, pers. comm.), and the offspring is kept at Dublin Zoo (Ireland) (n = 2) and at Kristiansand Zoo (Norway) (n = 1). In the European zoo community there is also another only recently genetically confirmed pair of *C. suchus* held in the Vivarium de Lausanne, which has deposited clutches in the past. Until recently, however, these animals were considered to be *C. niloticus* and therefore the clutches had never been incubated. A recent clutch, consisting of seven eggs, deposited in March 2014 at the Vivarium de Lausanne, has proved to be infertile (Michel Ansermet, pers. comm.), but further breeding attempts are planned for the future, both at the Vivarium de Lausanne and at Copenhagen Zoo.

In this study, we used mitochondrial DNA and one nuclear DNA marker (LDH-A) to assign specimens to either *C. niloticus* and *C. suchus.* While mtDNA reconstructs only the maternal genealogy, alleles of the two taxa in LDH-A differ only in a single mutation. It is obvious that the use of only two markers implies a low resolving power: while F1 hybrids would have probably been detected as heterozygotes in LDH-A, backcrosses can remain undetected. Besides a better geographic coverage and a larger number of samples we clearly require additional nuclear DNA markers to fully understand the distribution ranges of *C. suchus* and *C. niloticus*, the genetic structure of their contact zone, and possible gene flow between these two taxa. The need for additional nuclear markers extends to the specimens screened here, as exemplified by the Lebanon wild-caught individual held at Crocodiles of the World, which might represent natural introgression, or hybridisation in captivity and subsequent release in the wild. Microsatellite markers for crocodiles have been developed (FitzSimmons et al. 2001; Miles et al. 2009) and successfully applied to population genetics of *C. suchus* in Mauritania (Velo-Antón et al. 2014). The use of such high-resolution markers will be useful in obtaining a more reliable picture of gene flow between *C. niloticus* and *C. suchus*. However, compared to the previous situation in which the identity of Nile crocodiles in European zoos was completely uncharted, our study provides a first baseline for future captive breeding programmes.

The results of our extended genetic screening of *Crocodylus mindorensis* held in European zoos revealed all ten Philippine crocodiles now kept at the Protivin Crocodile Zoo (Czech Republic) to be pure *C. mindorensis*. These specimens were therefore also included in the European Studbook (ESB) for the Philippine crocodile (Ziegler et al. 2014), and thus the number of individuals and genetic diversity within the ESB population of *C. mindorensis* has been increased. As all ten Philippine crocodiles kept at Protivin Crocodile Zoo derive from a single, separately kept pair still being held at Avilon Zoo in the Philippines, our data also provide evidence that these two parents are pure *C. mindorensis*. With the data obtained from the genetic screening of Hauswaldt et al. (2013) and in the present paper we can be assured that all *C. mindorensis* kept within the ESB are purebred and can be employed for conservation breeding. The first successful breeding attempt of this species at a European facility took place at the Cologne Zoo (Ziegler et al. 2013), and was followed by additional success stories at Protivin Crocodile Zoo (Prochazka 2013), London Zoo (Gill 2014), and Danish Crocodile Zoo (see current overview in Ziegler and Rauhaus 2015). The purebred offspring are now available for zoos with expertise in crocodile husbandry that are interested in joining the Philippine crocodile conservation breeding programme.

Outlook

The genetic screening of crocodiles exemplified by *C. suchus* and *C. mindorensis* clearly illustrates the importance of applying

such methods for the proper husbandry management of zoos, in particular in the framework of conservation breeding programmes. As stated previously in Hauswaldt et al. (2013), we recommend routine use of genetic screening for taxa that are morphologically cryptic (see also Schmidt and Ziegler 2014), as well as for individuals that could potentially be of hybrid origin. We also advocate a stepwise procedure in which mtDNA and nuclear gene sequencing are first used to allocate individuals to species, because an immediate application of high-resolution markers such as microsatellites might not correctly identify single specimens that are misidentified at the species level.

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