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GENETIC SCREENING OF CAPTIVE PHILIPPINE CROCODILES (*CROCODYLUS MINDORENSIS*) AS PREREQUISITE FOR STARTING A CONSERVATION BREEDING PROGRAM IN EUROPE

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***Abstract.*—Philippine Crocodiles (*Crocodylus mindorensis*) are among the rarest crocodilians worldwide. Captive propagation for building up a conservation breeding and reserve population in Europe has recently been undertaken as a management action. For this purpose, 15 presumed *C. mindorensis* all originating from a captive source in the Philippines were brought to different facilities in Europe in 2006. Identification of hybrid individuals, deriving from crosses of the Philippine Crocodile with the Saltwater Crocodile (*C. porosus*) at a captive breeding facility in the Philippines prompted us to undertake a genetic screening of the European individuals to determine whether evidence for hybridization could be detected. We sequenced the 14 remaining *C. mindorensis* individuals, five additional *C. mindorensis* from other sources, and two *C. porosus* for two mitochondrial and three nuclear gene fragments. No evidence of *C. porosus* introgression was detected in 18 of the presumed pure *C. mindorensis*; however, we found one presumed pure *C. mindorensis* to be a Western Nile Crocodile (western lineage of *C. niloticus sensu lato*, proposed to be named *C. suchus* by Schmitz et al. 2003). Both *C. porosus* individuals were in genetic agreement with known *C. porosus* gene sequences. Of the three nuclear markers, LDH-A was most informative to discriminate between *C. mindorensis* and *C. porosus*. With this first genetic screening, an important step towards a proper European conservation breeding program has been made.**

Key Words.—*Crocodylus mindorensis*; *Crocodylus porosus; Crocodylus suchus*; hybridization; molecular screening; Western Nile Crocodile

INTRODUCTION

The Philippine Crocodile (*Crocodylus mindorensis*) was described by K.P. Schmidt based on type specimens from the Philippine island of Mindoro (Schmidt 1935, 1938). Subsequently, the Philippine Crocodile has long been treated as a subspecies of the New Guinea Freshwater Crocodile (*C. novaeguineae*), but recent taxonomic research based on morphological characters has provided new evidence for the distinct taxonomic status of *C. mindorensis* (Hall 1989). Morphologically *C. mindorensis* differs from *C. novaeguineae* in cervical squamation (e.g., prominent versus reduced nuchomarginal rows) and palatal structure; it is also distinctive with respect to the number of transverse dorsal midbody scales (12 versus 10) and in several aspects of the relative growth of the skull (Hall 1989), such as robust versus slender skull (snout length 1.6 times its basal width in *C. mindorensis* versus 2 times its basal width in *C. novaeguineae*), and more distinctly textured top of the head in *C. mindorensis* (see also Ross and Mayer 1983; Trutnau and Sommerlad 2006). Based on the findings of Hall (1989), the Philippine Crocodile henceforth has been treated as a full species endemic to the Philippines (van Weerd 2010). Previously widely distributed in the Philippines, the species now can be found only in small populations in south-western Mindanao and northern Luzon (van Weerd 2010). In 1998, the total natural population was estimated at fewer than 100 mature individuals (van Weerd et al. 2009). Thus, the Philippine Crocodile is among the rarest crocodilians in the world. The conversion of freshwater habitat, hunting, and the use of destructive fishing methods continue to threaten the remnant and fragmented populations of this species (van Weerd and van der Ploeg 2004; van de Ven et al. 2009).

Crocodylus mindorensis has been recognized by the Crocodile Specialist Group of the Species Survival Commission of the International Union for Conservation of Nature (IUCN) as one of the most threatened species of crocodiles in the world today. The species is listed as Critically Endangered by IUCN and on Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Crocodile Specialist Group 1996; UNEP-WCMC 2013). The Crocodile Specialist Group has placed the Philippine Crocodile on

top of its priority list for conservation action and strongly recommended *ex situ* management due to its fragile status in the wild (van Weerd 2010). Furthermore, the "National Recovery Plan for the Philippine Crocodile" recommends, in addition to *in situ* conservation, the coordination of the management of captive *C. mindorensis* in the Philippines and the establishment of a coordinated global captive management program for the species (Banks 2005). To increase the natural population, reintroductions already have taken place in the Philippines, conducted by the Mabuwaya Foundation based on captive bred individuals from the governmental Palawan Wildlife Rescue and Conservation Centre (PWRCC; e.g., van Weerd 2010).

Apart from captive management in the Philippines (e.g., Sumiller and Cornel 2008; van Weerd 2010), the species so far has been managed at seven facilities in North America (Gladys Porter Zoo, Texas; Omaha's Henry Doorly Zoo and Aquarium; Pittsburgh Zoo and Aquarium, Pennsylvania; Silver Springs Park, Florida; St. Augustine Alligator Farm, Florida; Tampa Lowry Park Zoo, Florida; and Smithsonian National Zoo, Washington, DC) under coordination of the Gladys Porter Zoo, at which the only breeding success so far has been recorded. In Australia, the management has been coordinated by Melbourne Zoo, which is the only zoo in the region keeping the species. In 2006, following the Philippine government's Philippine Crocodile National Recovery Plan, 15 Philippine Crocodiles from a Philippine breeding facility were transferred to zoos in Europe (Krokodille Zoo, Denmark; Cologne Zoo, Germany; Bergen Zoo, Norway; Chester Zoo [recently transferred from there to Paignton Zoo] and London Zoo, United Kingdom; and Zurich Zoo, Switzerland) in 2006 by the Danish "Krokodille Zoo." According to a Memorandum of Agreement, the transferred crocodiles (of which one died in the meantime at Bergen Zoo) remain the property of the Philippine government, and the hosting institutions are obliged to support the Mabuwaya Foundation, a small non-profit organization dedicated to the conservation of the species in its freshwater habitat (Banks et al. 2009; van Weerd 2010; van der Ploeg et al. 2011; Sommerlad et al. 2011). *Crocodylus mindorensis* has top priority in the regional collection plan of EAZA's (European Association of Zoos and Aquaria) taxon advisory group, and recently EAZA has established a conservation breeding program (European studbook, ESB) for the individuals kept in Europe which is managed by the Cologne Zoo (Ziegler et al. 2013).

Conservation genetic concepts have become a mandatory component of conservation breeding programs (Ouborg et al. 2006; Frankham et al. 2009), typically with the main goal to avoid or slow down inbreeding and to preserve a maximum of genetic diversity in a captive population (e.g., Leus et al. 2011; Witzenberger and Hochkirch 2011). Prior to establishing a conservation breeding program, however, it needs to be assessed whether all individuals belong to the same management unit or at least to the same species. For captive breeding with the intention of reintroduction it is pertinent to exclude inter-species hybrids (Allendorf et al. 2001; Fitzsimmons et al. 2002). In the genus *Crocodylus* this is an issue because hybridization, even among non-sister species, is common compared with other genera of Crocodylidae. In captivity, for example, hybrids between the Siamese Crocodile (*C. siamensis*) and the Saltwater Crocodile (*C. porosus*), have been observed (Chutharat Sukkhai et al. unpubl. report), as well as between the Siamese and the Cuban Crocodile (*C. rhombifer*; Thang 1994). In wild populations hybrids between Morelet's Crocodile (*C. moreletti*) and the American Crocodile (*C. acutus*) have been identified (Ray et al. 2004; Cedeno-Vazquez et al. 2008; Rodriguez et al. 2008), as well as between the Cuban and American Crocodiles (Milián-García et al. 2011). Also in *C. mindorensis* such concern is warranted because recent genetic screening with mitochondrial DNA (mtDNA) and nuclear markers of captive bred individuals in the Philippines considered for reintroduction identified a considerable number of morphologically indistinguishable hybrids between *C. mindorensis* and *C. porosus* (Tabora et al. 2012; John Aries Tabora, pers. comm.; Rheyda Hinlo, pers. comm.). Because these specimens did not exhibit obvious morphological differences to pure *C. mindorensis*, genetic screening presently seems to be the only method to reliably identify pure *C. mindorensis* for initiating conservation breeding and subsequent reintroduction programs. It needs to be emphasized that in this context, the definition of "pure" can only be relative. Many individuals of a given species might bear traces of ancient hybridization events in their genome, similar to the minute proportion of Neanderthal genes in many humans (Green et al. 2010). Such genomic patterns might nowadays even be seen as characterizing a species, and trying to remove them would be futile and unwarranted. What should be avoided for captive breeding programs, however, is the inclusion of recently originated hybrids which might even be human-induced, (e.g., through involuntary mixing of specimens in farms or through environmental changes that lead to the intrusion of one species into the habitat of the other, with subsequent hybridization).

To exclude such potential hybrid specimens from the conservation breeding program, we developed and conducted a comprehensive genetic screening of the crocodiles recently imported into Europe from Philippine breeding farms, by sequencing mtDNA as well as nuclear (nucDNA) gene fragments from every individual. We also included animals that had been separately imported from the Philippines to the Czech Republic in this study. Here

TTABLE 1. Captive facility and country abbreviation, morphological identification (Morph. ID), and source of specimens of *Crocodylus mindorensis, C. porosus*, and the Western Nile Crocodile for which DNA sequences were determined in our study: BFP = Philippine breeding facility, BFT = Breeding facility in Thailand, US-Phi = unknown source from Philippines. Numbers in first column correspond to specimen numbers used in the text and in Figure 1.

No.	Specimen	Sex	Facility	Morph. ID.	Source
1	CRNO	F	Bergen Zoo, NO	*C. mindorensis*	BFP
2	Mindo	F	Cologne Zoo, DE	*C. mindorensis*	BFP
3	Pinoy	M	Cologne Zoo, DE	*C. mindorensis*	BFP
4	GB1-6419	M	London Zoo, GB	*C. mindorensis*	BFP
5	GB2-6420	F	London Zoo, GB	*C. mindorensis*	BFP
6	GB3-12526a-b	F	Paignton Zoo, GB	*C. mindorensis*	BFP
7	GB4-12527a-b	M	Paignton Zoo, GB	*C. mindorensis*	BFP
8	Mindoro	M	Zoo Zurich, CH	*C. mindorensis*	BFP
9	Suba	F	Zoo Zurich, CH	*C. mindorensis*	BFP
10	Sulu	F	Zoo Zurich, CH	*C. mindorensis*	*
11	87244	juv.	Krokodille Zoo Eskilstrup, DK	*C. mindorensis*	BFP
12	92992	juv.	Krokodille Zoo Eskilstrup, DK	*C. mindorensis*	BFP
13	97518	juv.	Krokodille Zoo Eskilstrup, DK	*C. mindorensis*	BFP
14	97543	juv.	Krokodille Zoo Eskilstrup, DK	*C. mindorensis*	BFP
15	98148	juv.	Krokodille Zoo Eskilstrup, DK	*C. mindorensis*	BFP
16	2275518	juv.	Protivin Crocodile Zoo, CZ	*C. mindorensis*	US-Phi
17	2281357	juv.	Protivin Crocodile Zoo, CZ	*C. mindorensis*	US-Phi
18	23414019	juv.	Protivin Crocodile Zoo, CZ	*C. mindorensis*	US-Phi
19	Ocasek	juv.	Protivin Crocodile Zoo, CZ	*C. mindorensis*	US-Phi
20	Nunu	F	Wilhelma Zoo-Botanical Gardens, DE	*C. porosus*	BFT
21	Sue	F	Wilhelma Zoo-Botanical Gardens, DE	*C. porosus*	BFT

* originally kept at Zoo Dvur Kralove, Czech Republic, afterwards (since 1980) at the Zoo Wroclaw, Poland, from where it was recently transferred to Zoo Zurich. Specimen corresponding to the Western Nile Crocodile according to data presented herein, corresponding to *C. suchus* of Schmitz et al. (2003)

we present the first results of our molecular analyses and discuss the usefulness of our methodological approach.

MATERIAL AND METHODS

We extracted total genomic DNA from 21 crocodiles from blood or scute samples using proteinase K digestion (10 mg/mL) followed by a standard salt-extraction protocol (Bruford et al. 1992). Fourteen of the animals were putative *C. mindorensis* from a breeding facility in the Philippines, now housed at different European Zoos, four additional *C. mindorensis* from an unknown source from the Philippines, now housed at a Zoo in the Czech Republic, one putative *C. mindorensis* of unknown source housed at a zoo in Switzerland, and two *C. porosus* from a breeding facility in Thailand (Table 1). We amplified and analyzed two mitochondrial (12S rRNA and D-loop) and three nuclear gene segments (oocyte maturation factor [c-mos], cellular myelocytomatosis proto-oncogene (c-myc), and lactate dehydrogenase A [LDH-A] gene, exons 7–8 [LDHA]). We used published primers for all markers except for c-myc (see Table 2 for primer information). For this locus we designed new primers based on Genbank sequences EF646353–646359. At the onset of the study, we also tried amplifying Exon 5–6 of the α-tropomysin gene (aTROP) and Exon 11–12 of the glyceraldehyde 3 phosphate dehydrogenase (GAPDH) using the primers of Oaks (2011). However, polymerase chain reactions (PCR) yielded double bands and we discontinued using these markers. Each PCR contained a total volume of 12.5 µL, consisting of 1x PCR buffer, 0.24 µM of each primer, 200 µM dNTPs, and 0.4 units GoTaq (Promega, Mannheim, Germany; see Table 3 for PCR conditions). We treated PCR products with Exonuclease I (New England Biolabs, Ipswich, Massachusetts, USA) and Shrimp Alkaline Phosphatase (Promega) to inactivate remaining primers and dNTPs, and then sequenced with the amplification primers using dye-labeled terminators (Applied Biosystems).

TABLE 2. Primers used for PCR and cycle sequencing reactions and their respective references, size of amplicon, and length of sequence of each gene fragment.

Gene	Primer name and Sequence 3′ – 5′	Amplicon/Sequence length (bp)	Reference
12S rRNA	12SAL: AAACTGGGATTAGATACCCCACTAT 16SBHnew: CCTGGATTACTCCGGTCTGA	2033/400–700	Palumbi et al. 2002 RTPrimer DB*
D-Loop	t-PHE-L: GAACCAAATCAGTCATCGTAGCTTAAC CR2H: GGGGCCACTAAAAACTGGGGG	~656/~598	Ray and Densmore 2002
LDHA	LDHAI7-F1: TGGCTGAAACTGTTATGAAGAACC LDHAI7-R1: TGGATTCCCCAAAGTGTATCTG	743/697	Gatesy et al. 2004
c-mos	CmosF: ATAGTTGCTGTGAAGCAGGT CmosR: GCTCAGTGATGAACACATTG	388/347	Meganathan et al. 2010
c-myc	Cmyc-Croc-F: GGTGAATGGAGTTGAATCCGG Cmyc-Croc-R: AGCCAAGGTTGTGTAGTTGC	693/642	this study

* Real Time PCR Primer database (www.rtprimerdb.org)

Sequencing was done in both directions for nuclear genes and with the forward primer only for mitochondrial genes, on an ABI 3130XL automated DNA sequencer (Applied Biosystems, Carlsbad, California, USA). Using CodonCode Aligner (v. 2.0.6, CodonCode Corporation, Dedham, Massachusetts, USA), we checked chromatographs and edited and aligned sequences. We made alignments with all available Genbank sequences of *Crocodylus mindorensis*, *C. novaeguineae*, Freshwater Crocodile (*C. johnsoni*), *C. porosus*, *C. siamensis*, *C. palustris*), and the Nile Crocodile (*C. niloticus*) which included sequences of the Western Nile Crocodile (western lineage of *C. niloticus* sensu lato, proposed to be named *C. suchus* by Schmitz et al. 2003). We separated sequences of heterozygous individuals into haplotypes by eye as there was no sequence with more than one heterozygous position. We inferred haplotype networks based on the median-joining approach (Bandelt et al. 1999) as implemented in Network version 4.611 (www.fluxus-engineering.com). For this approach, the sequences had to be shortened to be of equal length, but we submitted the newly determined sequences in their original length to Genbank (for accession numbers, see Appendix Table).

RESULTS

Based on the haplotype networks of the mitochondrial markers (294 bp of 12S rRNA and 419 bp of D-loop), we could define all specimens clearly as *C. mindorensis*, except for the two putative *C. porosus* (#20 and #21), and one putative *C. mindorensis* from Zurich Zoo (#10; Fig. 1). While #20 and #21 grouped with other *C. porosus* as expected, #10's haplotypes were either shared with or closely related to those of *C. niloticus*, specifically to those of the western lineage (Western Nile Crocodile). Regarding the nuclear genes LDH-A (598 bp) and c-mos (248 bp), all specimens that had been assigned to *C. mindorensis* based on mtDNA also grouped with *C. mindorensis* sequences obtained from Genbank, #20 and #21 with sequences of *C. porosus*, and #10 with Western Nile Crocodile (Fig. 1). In these gene fragments, allele sharing was found between *C. novaeguineae* and *C. mindorensis*, but never between *C. mindorensis* and *C. porosus* or any other species. In fact, differentiation in LDH-A was particularly strong between *C. mindorensis* and *C. porosus* with seven substitutions (Fig. 1). In all other pairwise species comparisons there was at least one diagnostic site for this marker, except between *C. niloticus* (western lineage) and *C. siamensis* that shared an allele. Five of the putative *C. mindorensis* from the zoo collections were heterozygous at position 37, and thus identical with the sequence of individual #65; all other *C. mindorensis* shared the same allele. Alleles of c-mos were shared between the two Genbank sequences of *C. novaeguineae* and *C. mindorensis*, but again no evidence of hybridization with *C. porosus* was found, noting two diagnostic nucleotide substitutions. *Crocodylus porosus*, *C. siamensis*, and *C. niloticus* (eastern lineage), however, shared the same allele. According to the available *Crocodylus* Genbank sequences for c-myc, each species has one or two diagnostic bases. However, none of our 21 sequences (642 bp for all individuals except 523 bp only for #6, which was therefore not included in the network) matched with any of the sequences currently available in Genbank. We obtained four different haplotypes, and only two individuals (#20 and #21) were heterozygous (position 59). The haplotype of #10 differed by one mutation from the one of *C. niloticus*, however, it is not clear whether the Genbank sequence corresponds to a specimen from the

TABLE 3. PCR conditions used for amplification of different gene fragments.

	PCR steps	12S rRNA	D-loop	c-mos	c-myc	LDHA
1	Initial Denaturation (94°C) 2 min					
2	Denaturation (94°C)	45 s	120 s	60 s	60 s	45 s
3	Annealing	52°C 45 s	60°C 45 s	58°C 45 s	54°C 45 s	53°C 45 s
4	Extension (72°C)	90 s	80 s	80 s	180 s	60 s
5	Final Extension (72°C)	5 min	5 min	8 min	8 min	6 min
	Number of Cycles (2–4)	35	35	38	38	45

eastern or the western clade.

Our preliminary data reveal that individuals kept in European facilities represent *C. mindorensis* without any indication of a hybrid origin. Individuals #20 and #21 can be considered pure *C. porosus*, at least based on the mitochondrial markers and the one diagnostic nuclear marker for this taxon (LDH-A). Individual #10, on the other hand, is diagnosed as a pure Western Nile Crocodile based both on mitochondrial DNA and c-mos, the diagnostic nuclear marker for this taxon.

DISCUSSION

Our study used mtDNA and nucDNA markers to ascertain the taxonomic identity of the crocodile individuals studied as a first step towards the establishment of a captive breeding program of *C. mindorensis* in Europe. In general, given the incomplete knowledge on taxonomy and spatial population structure in most species of reptiles, applications of conservation genetic tools is frequently used for delimitation of species and definition of management units for conservation, as e.g. in Tuataras (*Sphenodon* spp., see DeSalle and Amato 2004) or Galapagos Tortoises (*Chelonoidis nigra*, e.g., Garrick et al. 2012). We identified all remaining specimens which originally came from a captive farm in the Philippines and were brought to Europe in 2006 as *Crocodylus mindorensis*, as well as the four animals from Protivin Zoo that potentially came from a different facility in the Philippines, as belonging to a single species and management unit, and we can exclude the possibility that any of them represent F1 hybrids with *C. porosus*. The two specimens of *C. porosus* (#20 and #21) also showed no evidence of hybrid origin. However, we cannot exclude the possibility that some parts of the nuclear genomes of any of the specimens might bear traces of more ancient hybridization with another species. While both mtDNA markers provide clear resolution among the species, we are aware that this is not the case for the nucDNA markers. Of the three nucDNA markers, LDH-A provides the strongest resolution among the taxa, in particular for distinguishing *C. mindorensis* and *C. porosus*, and c-myc the weakest. The need to quickly establish the set-up for the captive breeding of this species and to acquire the basic knowledge about the feasibility of our molecular screening, prompted us to accept some compromises in this respect. We are aware that more fine-scale genetic markers, such as DNA microsatellites, need to be used to determine the degree of relatedness among these individuals to select the optimal pairs for breeding. In fact, while such in-depth conservation genetic analyses are commonplace in captive colonies of mammals, they have only rarely been applied to reptiles despite the existence of the suitable molecular tools and their possible importance especially in species with small population sizes (e.g., Moore et al. 2008).

Most interestingly, one individual from Zurich Zoo (#10) proved to be a Western Nile Crocodile instead of *C. mindorensis*. This was somewhat surprising, in particular as there are distinct morphological differences between *C. mindorensis* and *C. niloticus sensu lato* (e.g., 3.1 versus 6.5 m maximum total length, snout length 1.6 times its basal width in *C. mindorensis* versus 2 times its basal width in *C. niloticus sensu lato*, and upper snout surface with longitudinal ridges in front of the eyes versus smooth snout surface before the eyes, which tends to become rugose in aged individuals, but never with longitudinal ridges, in *C. niloticus sensu lato*). The single specimen was originally kept at Zoo Wroclaw in Poland from 1980 onwards, after it had been acquired from the Czech Zoo Dvur Kralove, from where it was transferred to Switzerland in 2011. Unfortunately, the original source of this specimen is unknown. To our knowledge this is the first reported case of a Western Nile Crocodile currently held at a European zoo. Based on molecular evidence, this taxon from western and central Africa was only recently resurrected as a distinct species (*C. suchus*; Schmitz et al. 2003), albeit so far no formal taxonomic redescription has been made. More extensive molecular analyses (Hekkala et al. 2011; Meredith et al. 2011; Oaks 2011) rendered the Western

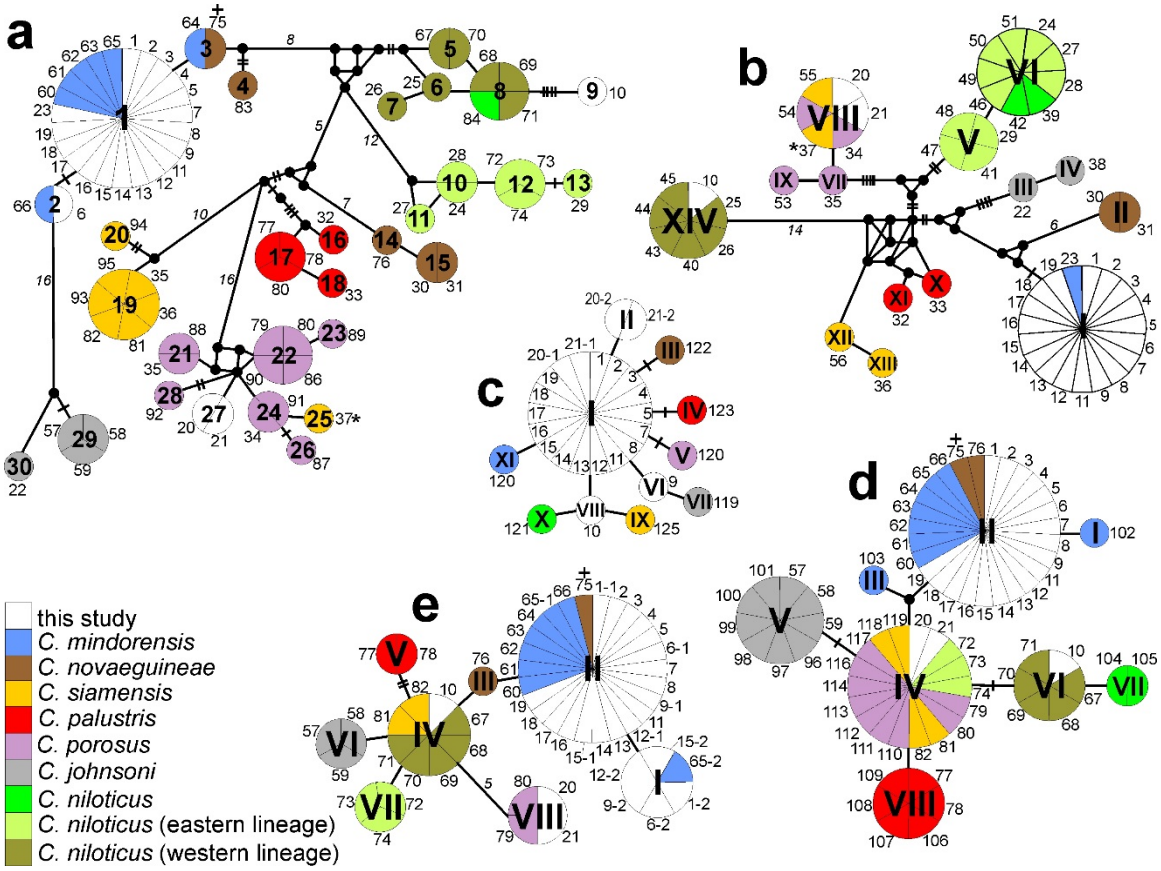

FIGURE 1. Median joining haplotype networks constructed on sequences of two mitochondrial (D-loop [a] and 12S rRNA [b]) and three nuclear gene sequences (c-myc [c], c-mos [d], and LDH-A [e]). The haplotype(s) of each individual is represented by a circle or a slice of a pie, and the number of each individual and its species assignment corresponds with Table 1 and the Appendix Table. Black circles indicate inferred haplotypes. Each connecting line between circles represents one mutational step. Up to four additional mutational steps are indicated by dashes and five or more steps by italicized numbers. *Crocodylus niloticus* (western lineage) also refers to lineage 2 of Oaks (2011) or Western Nile Crocodile, considered as *C. suchus* by Schmitz et al. (2003). For some individuals (e.g. #20 in c-myc), two haplotypes are shown, as these individuals were heterozygous. Individuals denoted by an asterisk (*) may be a hybrid of *C. siamensis* and *C. porosus* (Meredith et al 2011; Srikulnath et al. 2012) and those denoted by a cross (+) may actually be *C. mindorensis* according to Oaks (2011).

Nile Crocodile (lineage 2 according to Oaks 2011) sister to a clade containing the Eastern Nile Crocodile (*C. niloticus, sensu stricto*) and a subclade formed by the New World *Crocodylus*. A recent analysis of skull morphology also showed that western (Congo River Basin) *C. niloticus* (*sensu lato*) are significantly different from animals of Nile River or East African origin (Nestler 2012). The molecular identification of an adult individual of the Western Nile Crocodile among specimens studied by us has been crucial, as originally it was intended to be part of the *C. mindorensis* breeding program. In addition, it also represents a unique chance for starting up a separate conservation breeding program for the Western Nile Crocodile, given that this species is only poorly known and, due to its limited distribution, endangered as well. Further genetic screening will be required among small-growing adult *C. niloticus* in zoos to uncover potential Western Nile crocodiles for future pairing and breeding projects with #10, which currently is held separately in a behind-the-scenes facility at the Cologne Zoo.

Some of the Genbank sequences used in this study may have been derived from either misidentified or potentially hybrid individuals. For example, the mtDNA sequences of individual #37 (Ji et al. 2008), identified as *C. siamensis*, clearly group with *C. porosus*. Unfortunately, the authors did not provide locality information for this specimen. But already Meredith et al. (2011) and Srikulnath et al. (2012) suggested that this individual could be a hybrid of *C. siamensis* and *C. porosus*. For individual #55, also identified as *C.siamensis*, the 12S haplotype is identical with that of #37. Again, no locality information was provided and this animal may also be a hybrid with *C. porosus*. Interestingly, in c-mos, there seems to be true haplotype sharing between *C. porosus*, *C. siamensis* and *C.*

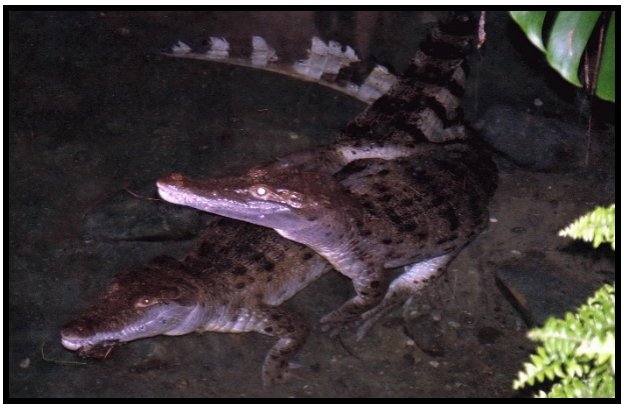

FIGURE 2. First copulation of Philippine Crocodiles (*Crocodylus mindorensis*) during the night of 3 February 2012 at the Cologne Zoo, Germany. (Photographed by Thomas Ziegler).

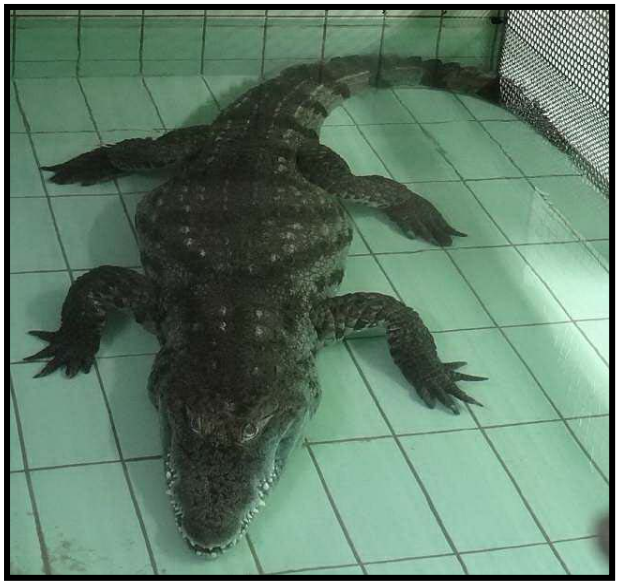

FIGURE 3. One of the "Philippine Crocodiles" from Zurich Zoo (#10; Sulu), Switzerland, which originally was kept at Zoo Wroclaw in Poland from 1980 onwards. It had been acquired from the Czech Zoo Dvur Kralove (from where it was transferred to Switzerland in 2011) and proved to be a Western Nile Crocodile (*Crocodylus suchus*) instead of a Philippine Crocodile (*C. mindorensis*). (Photographed by Anna Rauhaus).

niloticus (eastern lineage), as for all but two of these individuals evidence from other markers also is available, which clearly assigns these individuals to the respective species. Individual #75 (*C. novaeguineae*) which shares a D-loop, LDH-A, and c-mos allele with *C. mindorensis* originated from a captive facility in New Guinea and it is possible that it had been misidentified (Oaks 2011). It is however likely that the two sister taxa share an allele for c-mos, because individual #76 has the same c-mos allele as #75, but its D-loop sequence is quite distinct from that of #75 and differs by only one base from that of other *C. novaeguineae*.

With the first genetic screening of Philippine Crocodiles from European zoo facilities, an important step has been made towards a specifically tailored conservation breeding program for this species. Several additional Philippine Crocodiles kept at the Protivin facility, have not been screened yet, and we hope to include these individuals soon. We then will also conduct microsatellite analyses to get a finer resolution of the relationships among the individuals kept in Europe. With that information obtained, we will decide whether this stock is suitable for conservation breeding or whether additional individuals have to be imported from the Philippines to ensure sufficient genetic variability of the captive gene pool.

Crocodylus mindorensis is difficult to keep and housing requires careful preparation (e.g., Ziegler et al. 2011); thus, breeding is a challenging task. Whereas breeding in captivity frequently occurs in the Philippines and at one zoo in the US (Gladys Porter Zoo), there has been no breeding success so far in Europe. However, now most of the animals in Europe are mature and reproductive behavior has been noted in some institutions, such as in Cologne and Protivin.

Lastly, the genetic validation of *C. mindorensis* and the discovery of the *C. suchus* individual misidentified as *C. mindorensis* since first acquisition and maintained as such in multiple collections shows the importance of proper molecular identification of founder animals as a prerequisite for conservation breeding. Costs of DNA sequencing and other molecular methods have greatly decreased over the last decade and such procedures should become a prerequisite for conservation breeding, in particular if animals are eventually intended for reintroduction. Through the recently started "Cold Code" global DNA barcoding initiative for amphibians and non-avian reptiles (Murphy et al. 2013), at least mitochondrial sequences of the COI gene from unambiguously identified specimens will soon become available for the majority of species making such molecular surveys even easier. We recommend they should be conducted routinely for morphologically cryptic or difficult to identify taxa, as well as for individuals for which hybrid origin could be suspected.

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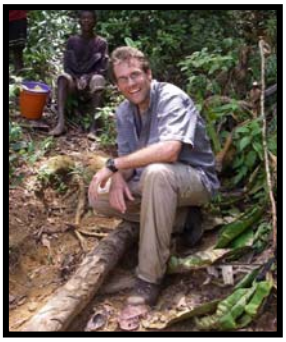

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APPENDIX TABLE. Genbank accession numbers of all individuals for the five different gene fragments. All *C. niloticus* marked with an asterisk belong to the Western lineage (Meredith et al. 2011; referred to as lineage 2 in Oaks 2011 and *C. suchus* in Schmitz et al. 2003).

No.	Individual	Inferred taxon	12S rRNA	D-loop	LDH-A	C-mos	c-myc
1	CRNO	*C. mindorensis*	KC849146	KC849167	KC849188	KC849209	KC849230
2	Mindo	*C. mindorensis*	KC849147	KC849168	KC849189	KC849210	KC849231
3	Pinoy	*C. mindorensis*	KC849148	KC849169	KC849190	KC849211	KC849232
4	GB1-6419	*C. mindorensis*	KC849149	KC849170	KC849191	KC849212	KC849233
5	GB2-6420	*C. mindorensis*	KC849150	KC849171	KC849192	KC849213	KC849234
6	GB3-12526	*C. mindorensis*	KC849151	KC849172	KC849193	KC849214	KC849235
7	GB4-12527	*C. mindorensis*	KC849152	KC849173	KC849194	KC849215	KC849236
8	Mindoro	*C. mindorensis*	KC849153	KC849174	KC849195	KC849216	KC849237
9	Suba	*C. mindorensis*	KC849154	KC849175	KC849196	KC849217	KC849238
10	Sulu	*C. niloticus**	KC849155	KC849176	KC849197	KC849218	KC849239
11	87244	*C. mindorensis*	KC849156	KC849177	KC849198	KC849219	KC849240
12	92992	*C. mindorensis*	KC849157	KC849178	KC849199	KC849220	KC849241
13	97518	*C. mindorensis*	KC849161	KC849179	KC849200	KC849221	KC849242
14	97543	*C. mindorensis*	KC849159	KC849180	KC849201	KC849222	KC849243
15	98148	*C. mindorensis*	KC849160	KC849181	KC849202	KC849223	KC849244
16	2275518	*C. mindorensis*	KC849158	KC849182	KC849203	KC849224	KC849245
17	2281357	*C. mindorensis*	KC849162	KC849183	KC849204	KC849225	KC849246
18	23414019	*C. mindorensis*	KC849163	KC849184	KC849205	KC849226	KC849247
19	Ocasek	*C. mindorensis*	KC849164	KC849185	KC849206	KC849227	KC849248
20	Nunu	*C. porosus*	KC849165	KC849186	KC849207	KC849228	KC849249
21	Sue	*C. porosus*	KC849166	KC849187	KC849208	KC849229	KC849250
22		*C. johnsoni*	NC_015238	NC_015238			
23		*C. mindorensis*	NC_014670	NC_014670			
24		*C. niloticus*	DQ273697	DQ273697			
25		*C. niloticus**	JF502243	JF502243			
26		*C. niloticus**	JF502244	JF502244			

APPENDIX TABLE. (Cont)

No.	Individual	Inferred taxon	12S rRNA	D-loop	LDH-A	C-mos	c-myc
27		*C. niloticus*	JF502245	JF502245			
28		*C. niloticus*	JF502246	JF502246			
29		*C. niloticus*	NC_008142	NC_008142			
30		*C. novaeguineae*	JF502240	JF502240			
31		*C. novaeguineae*	NC_015651	NC_015651			
32		*C. palustris*	HM488007	HM488007			
33		*C. palustris*	NC_014706	NC_014706			
34		*C. porosus*	DQ273698	DQ273698			
35		*C. porosus*	NC_008143	NC_008143			
36		*C. siamensis*	EF581859	EF581859			
37		*C. siamensis*	NC_008795	NC_008795			
38		*C. johnsoni*	AY195942				
39		*C. niloticus*	AY195943				
40		*C. niloticus**	AY195944				
41		*C. niloticus*	AY195945				
42		*C. niloticus*	AY195946				
43		*C. niloticus**	AY195947				
44		*C. niloticus**	AY195948				
45		*C. niloticus**	AY195949				
46		*C. niloticus*	AY195950				
47		*C. niloticus*	AY195951				
48		*C. niloticus*	AY195952				
49		*C. niloticus*	AY195953				
50		*C. niloticus*	AY195954				
51		*C. niloticus*	AY195955				
52		*C. palustris*	HM921182				
53		*C. porosus*	AY770534				
54		*C. porosus*	EU621800				
55		*C. siamensis*	AF237578				
56		*C. siamensis*	EU621801				
57	LSUMZ_H-21725	*C. johnsoni*		JF315345	JF315493	JF315195	
58	LSUMZ_H-21726	*C. johnsoni*		JF315383	JF315474	JF315176	
59	LSUMZ_H-7070	*C. johnsoni*		JF315381	JF315487	JF315189	
60	LSUMZ_H-21766	*C. mindorensis*		JF315349	JF315539	JF315242	
61	LSUMZ_H-21768	*C. mindorensis*		JF315364	JF315532	JF315235	
62	LSUMZ_H-21769	*C. mindorensis*		JF315342	JF315480	JF315182	
63	LSUMZ_H-21771	*C. mindorensis*		JF315341	JF315536	JF315239	
64	LSUMZ_H-21815	*C. mindorensis*		JF315333	JF315482	JF315184	
65	LSUMZ_H-21831	*C. mindorensis*		JF315343	JF315481	JF315183	
66	LSUMZ_H-21872	*C. mindorensis*		JF315332	JF315483	JF315185	
67	LSUMZ_H-21733	*C. niloticus**		JF315356	JF315501	JF315203	
68	LSUMZ_H-21734	*C. niloticus**		JF315355	JF315502	JF315204	
69	LSUMZ_H-21735	*C. niloticus**		JF315362	JF315503	JF315205	
70	LSUMZ_H-21736	*C. niloticus**		JF315361	JF315492	JF315194	
71	LSUMZ_H-21739	*C. niloticus**		JF315372	JF315515	JF315218	
72	LSUMZ_H-21731	*C. niloticus*		JF315377	JF315513	JF315216	
73	LSUMZ_H-21737	*C. niloticus*		JF315373	JF315530	JF315233	
74	LSUMZ_H-21738	*C. niloticus*		JF315334	JF315522	JF315225	
75	LSUMZ_H-6995	*C. novaeguineae*		JF315340	JF315494	JF315196	
76	LSUMZ_H-7071	*C. novaeguineae*		JF315331	JF315490	JF315192	
77	LSUMZ_H-21741	*C. palustris*		JF315359	JF315521	JF315224	
78	LSUMZ_H-21742	*C. palustris*		JF315358	JF315540	JF315243	
79	LSUMZ_H-6758	*C. porosus*		JF315369	JF315542	JF315245	

APPENDIX TABLE. (Cont)

No.	Individual	Inferred taxon	12S rRNA	D-loop	LDH-A	C-mos	c-myc
80	LSUMZ_H-6984	*C. porosus*		JF315353	JF315523	JF315226	
81	LSUMZ_H-6978	*C. siamensis*		JF315379	JF315471	JF315173	
82	LSUMZ_H-6985	*C. siamensis*		JF315360	JF315478	JF315180	
83		*C. mindorensis*		AF460209			
84		*C. niloticus*		AF460211			
85		*C. palustris*		AF460212			
86		*C. porosus*		AF460213			
87		*C. porosus*		AF542533			
88		*C. porosus*		AF542534			
89		*C. porosus*		AF542535			
90		*C. porosus*		AF542536			
91		*C. porosus*		AF542537			
92		*C. porosus*		AF542538			
93		*C. siamensis*		AF460215			
94		*C. siamensis*		AF542540			
95		*C. siamensis*		AF542542			
96		*C. johnsoni*				AY910608	
97		*C. johnsoni*				AY910609	
98		*C. johnsoni*				AY910610	
99		*C. johnsoni*				AY910611	
100		*C. johnsoni*				HM490317	
101		*C. johnsoni*				HM490318	
102		*C. mindorensis*				AY910620	
103		*C. mindorensis*				AY910621	
104		*C. niloticus*				AY910624	
105		*C. niloticus*				AY910625	
106		*C. palustris*				AY910612	
107		*C. palustris*				AY910613	
108		*C. palustris*				HM490313	
109		*C. palustris*				HM490314	
110		*C. porosus*				AF039484	
111		*C. porosus*				AF478196	
112		*C. porosus*				AY910622	
113		*C. porosus*				AY910623	
114		*C. porosus*				FJ011695	
115		*C. porosus*				HM490315	
116		*C. porosus*				HM490316	
117		*C. siamensis*				HM490319	
118		*C. siamensis*				HM490320	
119		*C. johnsoni*					EF646354
120		*C. mindorensis*					EF646355
121		*C. niloticus*					EF646359
122		*C. novaeguineae*					EF646356
123		*C. palustris*					EF646358
124		*C. porosus*					EF646357
125		*C. siamensis*					EF646353