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Genetic differentiation of the African dwarf crocodile *Osteolaemus tetraspis* Cope, 1861 (Crocodylia: Crocodylidae) and consequences for European zoos

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Abstract The endangered African dwarf crocodile *Osteolaemus tetraspis* is distributed in Central and Western Africa. Conventionally, two subspecies were distinguished: *Osteolaemus tetraspis tetraspis* and *Osteolaemus tetraspis osborni.* The taxonomic significance of diagnostic morphological characters is still being discussed and the existence of additional species in the *Osteolaemus* group remains unclear. Recent molecular studies suggest the existence of three allopatric species in the genus *Osteolaemus.* These results supported a division of the dwarf crocodile into a Congo Basin form (*O. osborni*), an Ogooué Basin form (*O. tetraspis*), and a third separate evolutionary lineage from Western Africa. Several European zoos host African dwarf crocodiles. For reasons of conservation and possible reintroduction, it is important to clarify provenance of these zoo animals. Therefore, we conducted molecular and phylogenetic analyses of three mitochondrial and two nuclear gene sequences with all available samples from European zoos and museums. We also estimated the origin of the zoo animals by comparing sequences of wild animals and museum samples of known provenance. Our study strongly supports three distinct lineages of *Osteolaemus* as recently postulated, but also reveals a fourth evolutionary lineage. We demonstrate that, of the European zoo animals sampled, only one dwarf crocodile corresponds to the Congo Basin form (*O. osborni*) whereas the majority of individuals correspond to the three other forms. Four zoo animals belong to the new fourth group; but their provenance is still unresolved. The origin of these animals is probably located in an African region from which no wild animal samples are currently available. Further investigations and sampling of other regions should be completed to clarify the identity of this fourth lineage. We found potential hybrids from European zoological gardens using nuclear DNA sequences. The European Studbook will use these results for further breeding programmes to keep genetically suitable ex-situ populations as reassurance colonies for prospective reintroduction into African countries.

Keywords African dwarf crocodile · *Osteolaemus* · Phylogenetic diversity · Cryptic species · European zoological gardens · European Studbook · Breeding programmes

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Introduction

African dwarf crocodiles (*Osteolaemus* spp.) are the smallest representatives of the family Crocodylidae and are distributed in Western and Central Africa (Trutnau and

Sommerlad 2006). The western distribution extends from Senegambia, Cameroon, Gabon down to North Angola. The Central African distribution includes the Central African Republic, Republic of Congo and Democratic Republic of Congo (Inger 1948; Trutnau and Sommerlad 2006). Two subspecies have been recognized: the Western African subspecies *Osteolaemus tetraspis tetraspis* and the Central African subspecies *Osteolaemus tetraspis osborni* (Wermuth 1953; Wermuth and Mertens 1961).

The endangered African dwarf crocodile is an important food source and economic resource for the rural human population in Central Africa (Zoer 2010) and is therefore hunted extensively. Its relatively small size and docility makes it an especially vulnerable target (Trutnau and Sommerlad 2006, Eaton et al. 2009a; Zoer 2010). Human population growth, modern hunting techniques and enhanced transportation infrastructure in Central Africa have led to increased commercial trade of dwarf crocodiles and other bushmeat species within the region (Eaton et al. 2009a; 2009b; Zoer 2010). *Osteolaemus* is registered as a vulnerable species in the Red List of IUCN and as an endangered species in the Washington Convention CITES (Convention of International Trade in Endangered Species, Appendix I species) because of exploitation and habitat loss (Crocodile Specialist Group 1996).

Taxonomic history of dwarf crocodiles

The taxonomy of dwarf crocodiles has been debated since the early part of the twentieth century (e.g., Schmidt 1919; Mertens 1943; Inger 1948; Ray et al. 2000; Brochu 2007; Eaton et al. 2009a). The first description of *Osteolaemus tetraspis* by Cope (Academy of Natural Sciences of Philadelphia 1860) was a specimen from the region of the Ogooué Basin. Almost 60 years later, Schmidt described a new genus with one species *Osteoblepharon osborni (Schmidt 1919)*.

This specimen was collected during a herpetological field trip to the Ituri Forest in the former Belgian Congo—now an area of the modern Democratic Republic of Congo (Eaton et al. 2009a). Schmidt (1919) distinguished two genera of dwarf crocodiles by morphological characters. He found differences in regions of the skull, e.g., the form of the snout, the missing nasal bone and straighter palatine bone of *Osteoblepharon* in contrast to *Osteolaemus*. However, he also stated that both genera are difficult to distinguish based on external morphological characters. Subsequently, *Osteoblepharon* was rejected as an invalid genus by a number of authors (e.g., Kälin 1933; Mertens 1943; Inger 1948), and as such regarded as a synonym of *Osteolaemus*. Some authors rejected differences between *Osteolaemus tetraspis* and *Osteolaemus osborni* as being intraspecific or ontogenetic, and consequently all described species of the genus *Osteolaemus* were synonymized with *Osteolaemus tetraspis*. Two geographically separated subspecies were described: *Osteolaemus tetraspis osborni* from the Congo Basin and *Osteolaemus tetraspis tetraspis* from Western Africa (Wermuth 1953; Wermuth and Mertens 1961). The taxonomic validity of subspecies within *Osteolaemus* is still under debate (King and Burke 1989; Ray et al. 2000; Ross 2006). However, a recent molecular study using mitochondrial and nuclear gene fragments [cytochrome-*c* oxidase (COI), 12S rDNA (12S), cytochrome b-tRNA threonine, proline, phenylalanine-control region (cytb), lactate dehydrogenase A (LDH-A) and recombination-activating gene 1 (RAG-1)] provides persuasive evidence for the existence of three allopatric, cryptic species within *Osteolaemus* (Eaton et al. 2009a).

The investigated samples originated from (1) wild populations in National Parks in the Republic of Congo and Gabon, (2) animals in the wild and in zoological gardens in Ghana and Côte d´Ivoire, (3) dried skin samples from the holotype and the paratype of *Osteolaemus tetraspis* osborni (Schmidt 1919) collected in the Democratic Republic of Congo (DRC), and (4) a sample collected in Cameroon (Eaton et al. 2009a). Phylogenetic analyses of wild animals suggested a division of African dwarf crocodile into a Congo Basin form (*O. osborni*), an Ogooué Basin form (*O. tetraspis*) and a third separate evolutionary lineage from Western Africa (*O.* sp. nov.). A molecular study by Eaton et al. (2009a) demonstrated high genetic divergence between the three evolutionary lineages, constituting three allopatric populations. Although the samples collected by Eaton et al. (2009a) precluded definitive identification of phylogeographic barriers, they hypothesized that the Batéké Plateau may serve to separate the Ogooué from the Congo Basins and that either the Dahomey Gap (a dry region between Togo and Benin) or the Cameroon-Nigerian Highlands acts as a barrier between West Africa and the Ogooué Basin (Fig. 1); analysis of mitochondrial sequences of COI—a barcoding gene—revealed a sequence divergence of 10–16 % (Eaton et al. 2009a). Based on these results, a complete revision of the taxonomy of *Osteolaemus* was conducted (Shirley and Eaton 2010), and the existence of three distinct phylogenetic species was suggested. These results have been accepted in recent compilations of phylogenetic analyses within the family of Crocodylidae (Oaks 2011).

Ex situ conservation efforts

Several zoological gardens host African dwarf crocodiles. It is important to be aware of the provenance of the zoo animals for conservation and possible reintroduction projects. For the species *Osteolaemus tetraspis*, the European Association of Zoos and Aquaria (EAZA) has established the European Studbook (ESB), in which European zoological gardens collaborate to record breeding programmes and

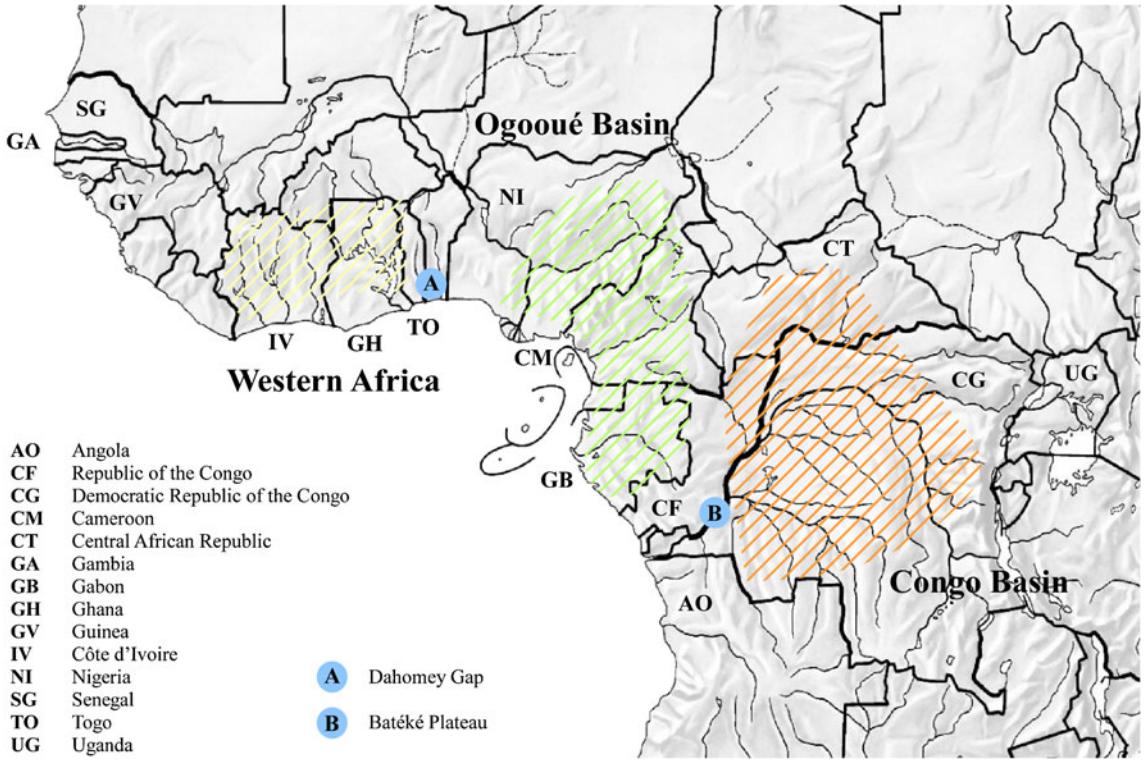


Fig. 1 Distribution map of African dwarf crocodiles according to the results of Eaton et al. (2009a) and this study. The three divergent evolutionary lineages are *shaded* (Western Africa, Ogooué Basin, Congo Basin). The geographical barriers are indicated with *circles A* and *B*. Basiskarte: © SWISS WORLD ATLAS 2010–2012

coordinate the breeding of threatened and endangered species, such as the African dwarf crocodile in European zoos. Maximizing the genetic viability of ex situ populations requires managed breeding to prevent hybridization and inbreeding. The aim of such breeding programmes is to sustain viable ex situ populations as reassurance colonies for prospective reintroduction to appropriate localities corresponding to the origins of the zoo animals.

The provenance of zoo animals is not always known and can sometimes only be partly proven due to fragmentary official papers. Also, several zoo animals are confiscated at customs without a known origin, and breeding in private institutions may obscure the origin of individuals of a species. Currently, 163 dwarf crocodiles are hosted in 58 European institutions and breeding has been successful. Because of the long lifespan of crocodiles, a majority of founders of the zoo population (wild animals) are still alive. The aim of the present study was to investigate the taxonomic status of African dwarf crocodiles in European zoological gardens and private institutions based on molecular methods. We investigated 75 zoo animals, including one wild animal from Port Harcourt (Nigeria) and animals from private breeders. To reconstruct the origin of the zoo animals we examined all available tissue samples of *Osteolaemus* from different museums and also included the sequences of wild animals generated by Eaton et al. (2009a) in our analyses. In accordance with Eaton et al. (2009a), we analyzed sequences of three mitochondrial genes and two nuclear genes: COI, 12S and cytb/CR and regions of the nuclear genes LDH-A and RAG-1. We estimated the genetic variation of zoo and wild-captured animals and determined hybrids between genetically distinct evolutionary lineages, which are a common phenomenon in zoos (Van Bemmel 1971; Dathe 1978; Lacy 1991; Marker-Kraus and Grisham 1993; Wangchuk et al. 2008). The European Studbook will use these results for a re-coordination of the zoo animals in Europe to avoid any hybridization and maintain distinct lineages in the ex-situ reassurance colony to provide genetically suitable animals if reintroduction programmes are needed.

Materials and methods

Sampling, DNA-extraction, amplification, DNA-sequencing and alignment

Blood samples from African dwarf crocodiles of zoological gardens in Europe and Toronto, Canada, and tissue samples from different museums were collected (see Supplementary material S1, S2). Samples were preserved in either 70 % ethanol or in thymol- or sodium ethylenediamine-tetraacetaic

acid (EDTA), and stored at −21° Celsius. Genomic DNA was extracted using a NucleoSpin®-Blood-Kit or NucleoSpin®-Tissue-Kit (Macherey-Nagel, Düren, Germany), following the manufacturer's protocol. From the genomic DNA template, we amplified and sequenced fragments of mitochondrial COI, 12S and cytb/CR, and nuclear LDH-A and RAG-1 genes. Internal primers were required for sequencing RAG-1 gene fragments and for COI fragments of degraded museum samples (see Table 1 for primer sequences and see Supplementary Material S3). We selected these genes for our investigation to allow us to include data from, and compare our results with those of, Eaton et al. (2009a).

Polymerase chain reactions (PCR) were performed in a volume of 20 µl and contained 2.0 µl genomic DNA (~20 ng/µl), 1 x PCR Buffer (Dream Buffer, Fermentas, St. Leon-Rot, Germany), 2 µl 2 mM dNTPs, 0.5 Unit *Taq* polymerase (Dream *Taq*, Fermentas) and 1 µl of each forward and reverse primer (Invitrogen, Karlsruhe, Germany). Amplification was performed in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany).

The cycling for COI (CoxIL2/CoxIH2) was set up with a first denaturation step at 95 °C for 5 min, followed by 35 cycles including denaturation at 95 °C for 1 min, annealing at 50 °C for 1.5 min and elongation at 72 °C for 1 min. PCRs using published primers followed thermocycling conditions provided in the original publications (Table 1). PCR products were purified using NucleoSpin® Extract II-Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocols.

All gene regions were sequenced in both directions using Big Dye™ Terminator v. 3.1 Cycle Sequencing Ready Reaction (Applied Biosystems, Warrington, UK) and visualized on an ABI PRISM 3100 (Applied Biosystems). Forward and reverse sequences were assembled into contigs, edited with BioEdit 7.0.5.3 (Hall 1999) and verified by eye. Contig sequences were aligned using the online version of MAFFT (Katoh et al. 2005), applying the FFT-NS-i strategy.

We identified heterozygous sites in nuclear sequences based on double peaks in the chromatograms. All sequences of each investigated African dwarf crocodile were submitted to GenBank under accession nos. JX626978 – JX627246 (Supplementary material S1 and S2).

We assembled three different datasets, a dataset of 3,748 aligned nucleotide positions for 67 taxa combining COI, cytb/CR, LDH-A, and RAG-1 (multilocus phylogenetic analysis); a dataset of 564 aligned nucleotide positions for 114 taxa (including additional museum samples and identified putative hybrid samples) using COI; and a dataset of 4,148 aligned nucleotide positions for 31 taxa combining all investigated genes (COI, cytb/CR, 12S, LDH-A, and RAG-1). For all analyses, the Nile crocodile (*Crocodylus*

Table 1 Primers used in this study. COI Cytochrome c oxidase; cytb/CR cytochrome b-tRNA threonine, proline, phenylalanine-control region; LDH-A lactate dehydrogenase A, RAG-1 recombination-activating gene 1

Gene	N[a]	Size[b]	Primer	Sequence	Source
12S	29	400	12SA-L	AAACTGGGATTAGATACCCCACTAT	Schmitz et al. (2003)
			12SB-L	GAGGGTGAACGGGCGGTGTGT	Schmitz et al. (2003)
COI	75	564	CoxIL2	GGCTACTGCCACTAATAATCG	This study
			CoxIH2	CCTAAGAAGCCAATTGATATTATG	This study
			MPCoF1	CGCCTTCATTGAAACAGGCGC	This study
			MPCoxF1	CACCACTTTTCGTATGATCCG	This study
Internal Primer for COI			MPCoF2	CGCCCTACCGGTCCTAGCGGCA	This study
			MPCoR1	CAGCTAGTGGTGGGTAAAC	This study
			P3CoxR1	CGGATCATACGAAAAGTGGTG	This study
			MPCoR3	GGATGTAGACTTCTGGGTGGC	This study
Cytb/CR	75	753	14943L	CCRTTYCACCCATACTTCTC	Eaton et al. (2009a)
			15789H	GGGTACATATTATCTTTYAMT	Eaton et al. (2009a)
LDH-A	75	655	LA17-F1	TGGCTGAAACTGTTATGAAGAACC	Gatesy et al. (2004)
			LA17-R1	TGGATTCCCCAAAGTGTATCTG	Gatesy et al. (2004)
RAG-1	75	1776	RAGL1	ACTCGATTTTGTCACAATTG	Gatesy et al. (2004)
			RAGR1	ATAGCTTCCAGCTCATCTGCTTG	Gatesy et al. (2004)
			RAGL3	AAGGCTGTTTGCATGACTTTGTT	Gatesy et al. (2004)
			RAGR5	AGCAAAGTTTCCATTCATCCTCAT	Gatesy et al. (2004)

[a] Number of sequences for each gene fragment.

[b] Size (in bases) of analysed gene fragment

niloticus) and slender-snouted crocodile (*Mecistops cataphractus*) were used as outgroup taxa (see Supplementary material S4). In addition to the sequence data we generated from zoo animals and museum samples, we included sequences of dwarf crocodiles sampled in situ (Eaton et al. 2009a; M. Eaton personal communication) in our phylogenetic analysis to determine the putative geographic origins of the ex situ samples (Supplementary material S5).

Data analyses

As in Eaton et al. (2009a), we calculated corrected genetic distances between and within clades (each lineage in Fig. 2, zoo animals only) for all genes (K2P, Kimura 1980) included in this study separately with MEGA 4.0 (Tamura et al. 2007). Four methods of phylogenetic inference were then used for all datasets: Neighbor-joining (NJ), maximum-parsimony (MP), maximum-likelihood (ML), and Bayesian inference (BI).

For MP analysis, conducted in PAUP 4.0b10 (Swofford 2002), heuristic searches were carried out using the tree bisection and reconnection (TBR) algorithm with 1,000 replicates. Taxa were added randomly using stepwise addition. Maximum likelihood (ML) was conducted using RAxML version 7.0.3 (Stamatakis 2006), choosing the GTR + G + I model using a partitioned dataset, allowing optimization of model parameters for each gene partition separately. NJ analyses were conducted in PAUP 4.0b10 (Swofford 2002) using the GTR-model (Rodriguez et al. 1990) and assuming a gamma-shaped distribution of rate variation among sites. Nodal support was calculated using 1,000 bootstrap replicates for ML, NJ, and MP (Felsenstein 1985). We used MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003; Altekar et al. 2004) to perform Bayesian inference of phylogeny using the GTR + G + I model. The analysis was run twice with four Metropolis-coupled Markov chains sampled every 500 generations for 1 million generations (MCMCMC, Huelsenbeck and Ronquist 2001). An average standard deviation of split frequencies of<5 % was used to indicate convergence in scores across chains. Based on this value, approximately the first 10 % of sampled trees were discarded as "burn-in" for all analyses. Remaining trees were used to construct the majority-rule consensus tree and posterior probabilities. All trees were visualized in TreeView version 1.6.6 (Page 1996) and iTol version 2.1.1 (Letunic and Bork 2006, 2011). Statistical parsimony networks for mitochondrial and nuclear sequences were constructed using TCS v.1.21 (Clement et al. 2000). We used the TCS default of a 95 % parsimony connection limit for conducting haplotype networks. Gaps were assumed as missing data.

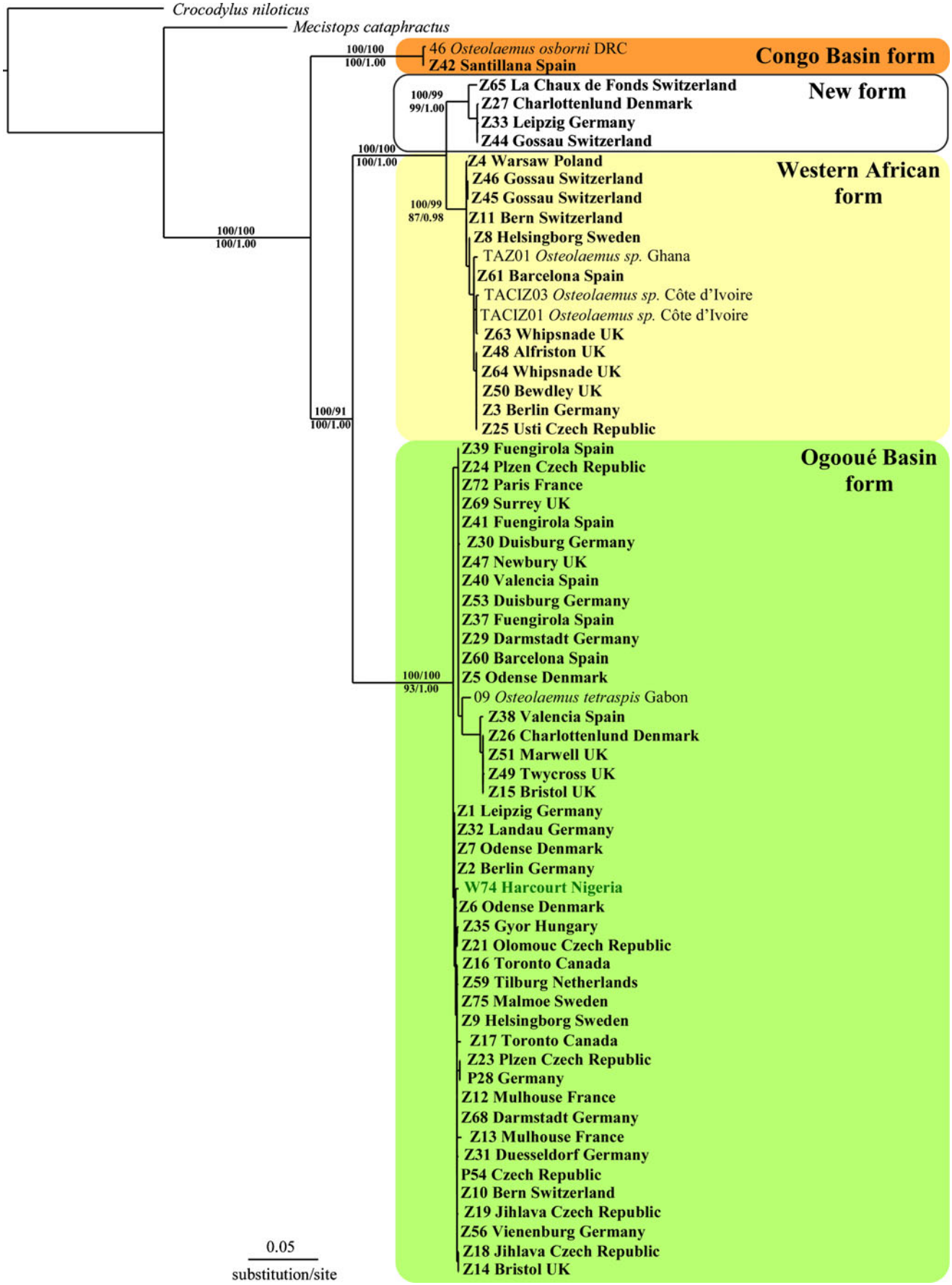
Results

We sequenced 75 individuals from zoos, covering both of the mitochondrial DNA regions (COI and cytb/CR) and both of the nuclear gene regions (RAG-1 and LDH-A). Additionally, we sequenced the mitochondrial 12S rDNA gene from 29 of these 75 individuals for an analysis with all five gene sequences. We also sequenced a part of the COI gene from museum samples and compared them with sequences of zoo and wild animals.

We were able to identify hybrids between the African dwarf crocodiles from European zoos by comparing the chromatograms of the nuclear gene sequences and those of the parental sequences (data not shown). In total we found 15 putative hybrids. As expected, divergent sequences from the parents of hybrids confirmed their origins from different lineages. Moreover, a hybrid origin is further confirmed by the fact that identified double detections of nucleotides were always found in informative positions (i.e., those positions that differ between clades) within the sequences of the investigated nuclear gene fragments (LDH-A, RAG1). All hybrids detected were captive bred. No heterozygous sites were observed in the nuclear genes of wild-caught crocodiles.

After excluding hybrids from our dataset, a total of 60 African dwarf crocodiles from zoos and other institutions remained. Phylogenetic analyses of all investigated gene fragments of these dwarf crocodiles result in four, well-supported monophyletic groups (Fig. 2). The Congo Basin form is resolved as the sister group to the remaining dwarf crocodiles. Within these, the Ogooué Basin form represents the sister group to a new lineage of *Osteolaemus*, which comprised two well-supported reciprocally monophyletic groups. One of these groups corresponds to the Western African form *sensu* Eaton et al. (2009a). This topology is recovered in all phylogenetic analyses and major clades are well supported by high bootstrap values and Bayesian posterior probabilities. Crocodiles from zoological gardens were identified from all four phylogenetic groupings, suggesting that European collections contain representative dwarf crocodiles from all known phylogeographic regions of Central and West Africa.

Only one investigated zoo animal grouped within the Congo Basin form: a male dwarf crocodile from the Santillana Zoo in Spain. The majority of the animals investigated group within the Ogooué Basin form (Table 2). Twelve zoo individuals belong to the Western African lineage, which included specimens from Ghana and Côte d´Ivoire. The fourth clade ("New Form" in Fig. 2), represented by four zoo individuals, could not be attributed to a geographic region due to a lack of provenance data. Sequences from a wild-caught dwarf crocodile from Harcourt, Nigeria (W74) clustered with animals of the Ogooué Basin form, as


Crocodylus niloticus
Mecistops cataphractus
100/100
100/1.00
46 Osteolaemus osborni DRC
Z42 Santillana Spain
Congo Basin form
Z65 La Chaux de Fonds Switzerland
Z27 Charlottenlund Denmark
Z33 Leipzig Germany
Z44 Gossau Switzerland
100/99
99/1.00
New form
100/100
100/1.00
Z4 Warsaw Poland
Z46 Gossau Switzerland
Z45 Gossau Switzerland
100/99
87/0.98
Z11 Bern Switzerland
Z8 Helsingborg Sweden
TAZ01 Osteolaemus sp. Ghana
Z61 Barcelona Spain
TACIZ03 Osteolaemus sp. Côte d'Ivoire
TACIZ01 Osteolaemus sp. Côte d'Ivoire
Z63 Whipsnade UK
Z48 Alfriston UK
Z64 Whipsnade UK
Z50 Bewdley UK
Z3 Berlin Germany
Z25 Usti Czech Republic
Western African form
100/100
100/1.00
100/91
100/1.00
Z39 Fuengirola Spain
Z24 Plzen Czech Republic
Z72 Paris France
Z69 Surrey UK
Z41 Fuengirola Spain
Z30 Duisburg Germany
Z47 Newbury UK
Z40 Valencia Spain
Z53 Duisburg Germany
Z37 Fuengirola Spain
Z29 Darmstadt Germany
Z60 Barcelona Spain
Z5 Odense Denmark
100/100
93/1.00
09 Osteolaemus tetraspis Gabon
Z38 Valencia Spain
Z26 Charlottenlund Denmark
Z51 Marwell UK
Z49 Twycross UK
Z15 Bristol UK
Z1 Leipzig Germany
Z32 Landau Germany
Z7 Odense Denmark
Z2 Berlin Germany
W74 Harcourt Nigeria
Z6 Odense Denmark
Z35 Gyor Hungary
Z21 Olomouc Czech Republic
Z16 Toronto Canada
Z59 Tilburg Netherlands
Z75 Malmoe Sweden
Z9 Helsingborg Sweden
Z17 Toronto Canada
Z23 Plzen Czech Republic
P28 Germany
Z12 Mulhouse France
Z68 Darmstadt Germany
Z13 Mulhouse France
Z31 Duesseldorf Germany
P54 Czech Republic
Z10 Bern Switzerland
Z19 Jihlava Czech Republic
Z56 Vienenburg Germany
Z18 Jihlava Czech Republic
Z14 Bristol UK
Ogooué Basin form
0.05
substitution/site

◀ **Fig. 2** Phylogenetic reconstruction using RAxML analysis of mitochondrial gene fragments [cytochrome *c* oxidase (COI); cytochrome b-tRNA threonine, proline, phenylalanine-control region (COI, cytb/CR)] and nuclear gene fragments [lactate dehydrogenase A (LDH-A), recombination-activating gene 1 (RAG1)] based on 60 African dwarf crocodiles from European zoos, a Canadian zoo, Nigeria and private breeders and sequences from wild animals (Eaton et al. 2009a). Numbers at nodes correspond to neighbor joining/maximum parsimony (NJ/MP) bootstraps above the branch, and maximum-likelihood (ML) bootstrap/Bayesian posterior probabilities below. NJ, MP and Baysian inference (BI) trees are not shown. *Crocodylus niloticus* and *Mecistops cataphractus* were used as outgroup taxa. The abbreviations represent the provenance of the animals (*Z* zoo animal, *W* wild animal, *P* animals from private breeders). The dwarf crocodile sequences produced in this study are in *bold*; other sequences are from GenBank (Eaton et al. 2009a)

did two zoo individuals with provenance in Nigeria (Z12 and Z14).

All phylogenetic analyses, except the concatenated nuclear dataset (Supplementary material S6) support the existence of four evolutionary lineages within *Osteolaemus* (Fig. 2, Supplementary material S7, S8, S9). The analysis of the concatenated nuclear gene fragments reveals a division of *Osteolaemus* lineages into Congo Basin, Ogooué Basin and Western African form (Supplementary material S6).

Because museum collections often include data on provenance, we sequenced a portion of the COI region from 16 dwarf crocodiles sampled from European museums to include as additional support for our phylogeographic analysis (Supplementary material S2). In total, we reconstructed a COI phylogenetic tree for 114 specimens to compare the origin of the museum samples (only COI sequences available) with the newly investigated zoo animals. The COI analysis included sequences of individuals from zoos, museums, private breeders, wild animals and outgroups. The analyses of COI of all the investigated individuals reveal the same four well-supported, separate evolutionary lineages within *Osteolaemus* (Supplementary material S9). According to this topology, an unnamed lineage is divided into two forms (i.e., Western African form and "new form") supported by high bootstrap values. These two monophyletic groups are supported by bootstrap values higher than 90 % and Bayesian posterior probabilities of 1.00. Similar to the multilocus analyses, the majority of the animals are grouped to the Ogooué Basin form (70 out of 91 zoo and museum samples). The other lineages contain the remaining animals, whereby seven animals belong to the new form of unknown origin (including the three hybrid sequences). One of the museum samples (M4 from Central Africa) clusters into the Congo Basin form and another sample (M1 from Togo) into the Western African form, which corresponds to the description of their geographic origin. The sequences of the remaining museum samples clustered within the Ogooué Basin form. The origin of these samples includes regions from the Ogooué Basin, but also regions from Western Africa, e.g. Liberia and Sierra Leone.

Table 2 Record of the *Osteolaemus* lineages and the corresponding number of zoo animals investigated in our combined phylogenetic analysis (Fig. 2)

Osteolaemus lineages	Number of zoo animals (total 60)
Congo	1
New form	4
Western Africa	12
Ogooué	43

We calculated K2P distances of the mitochondrial and nuclear gene fragments between and within the four *Osteolaemus* lineages for all zoo animals, excluding the museum samples (Table 3). We compared the results of this distance analysis for zoo crocodiles to those of wild-caught animals provided by Eaton et al. (2009a). The mitochondrial genes clearly exhibit greater genetic distances than the nuclear genes. After grouping captive samples by their respective clades (see above) K2P-distances for both nuclear genes and mitochondrial genes were of similar magnitude to the levels of divergence found for wild samples collected in situ by Eaton et al. (2009a). The genetic distances in the mitochondrial gene fragments between the Western African form and the new form with unknown origin range from 1.8 up to 4.2 %. Within lineages, the genetic distances are always below 0.8 %.

We conducted haplotype network analyses for concatenated mtDNA sequences (12S, COI, cytb/CR) and nuDNA sequences (LDH-A, RAG1) separately. In both analyses we applied a parsimony connection limit of 95 %. The haplotypes of 33 individuals were included in the network analyses. The analysis of the more conservative nuclear gene sequences reveals only one network with three clearly distinguishable groups (Fig. 3a). The three individuals from the new form with unknown origin are grouped together into the Western African lineages. The analysis of mitochondrial gene fragments resulted in four separate networks, which correspond to the four distinct evolutionary lineages of the phylogenetic reconstructions (Fig. 3b).

Discussion

Phylogeny and genetic variation within *Osteolaemus*

Our phylogenetic analyses confirm the presence of distinct evolutionary lineages of African dwarf crocodiles as reported by Eaton et al. (2009a). Field investigations and phylogenetic analyses by Eaton et al. (2009a) suggested a division of *Osteolaemus* into a Congo Basin form (*O. osborni*), an Ogooué Basin form (*O. tetraspis*), as well as a third, separate evolutionary lineage from Western Africa (*O.* sp. nov.). Additionally, our phylogenetic analyses of the combined and mitochondrial datasets support a fourth

Table 3 K2P-distances of gene sequences between and within the divergent evolutionary lineages of African dwarf crocodiles in European zoos. According to the phylogenetic tree in Fig. 2, we calculated the genetic distances for COI, cytb/CR, LDH-A and RAG-1 using Kimura 2-parameter and sequences of 60 zoo and 5 wild animals. For calculation of genetic distances in 12S, we used sequences of 29 zoo and 5 wild animals (Supplementary Fig. S8)

Osteolaemus lineages	12SrDNA	COI	cytb/CR	LDH-A	RAG-1
Congo vs. Ogooué	0.046	0.083	0.143	0.004	0.004
Congo vs. Western Africa	0.044	0.110	0.139	0.004	0.005
Congo vs new	0.044	0.108	0.137	0.004	0.005
Ogooué vs Western Africa	0.021	0.080	0.122	0.000	0.001
Ogooué vs new	0.008	0.094	0.137	0.000	0.001
Western Africa vs new	0.018	0.028	0.042	0.000	0.000
Within Congo	0.000	0.002	0.000	0.000	0.000
Within Ogooué	0.002	0.002	0.003	0.000	0.000
Within Western Africa	0.001	0.001	0.003	0.000	0.000
Within new	0.008	0.004	0.006	0.000	0.000

evolutionary lineage, which constitutes a sister group to the Western African form.

However, analysis of nuclear gene sequences does not reveal a separation of a fourth lineage (Supplementary material S6). Although the animals of this fourth lineage are grouped together, they cluster within the Western African form. There may be different reasons for this: generally, mitochondrial DNA exhibits faster substitution rates than nuclear DNA (Brown et al. 1979; Wilson et al. 1985; Saccone et al. 1999). This is due mainly to lower effective population size, haploidy and lack of recombination (Ballard and Whitlock 2004). In addition, the investigated nuclear genes, especially the nuclear gene RAG1, contain less genetic information for clarification of recent events of speciation (Janke et al. 2005). Unsurprisingly, the genetic distances of the investigated mitochondrial genes are much higher than the distances of the nuclear genes (Table 3). COI, which is used as a standard to identify species (Hebert

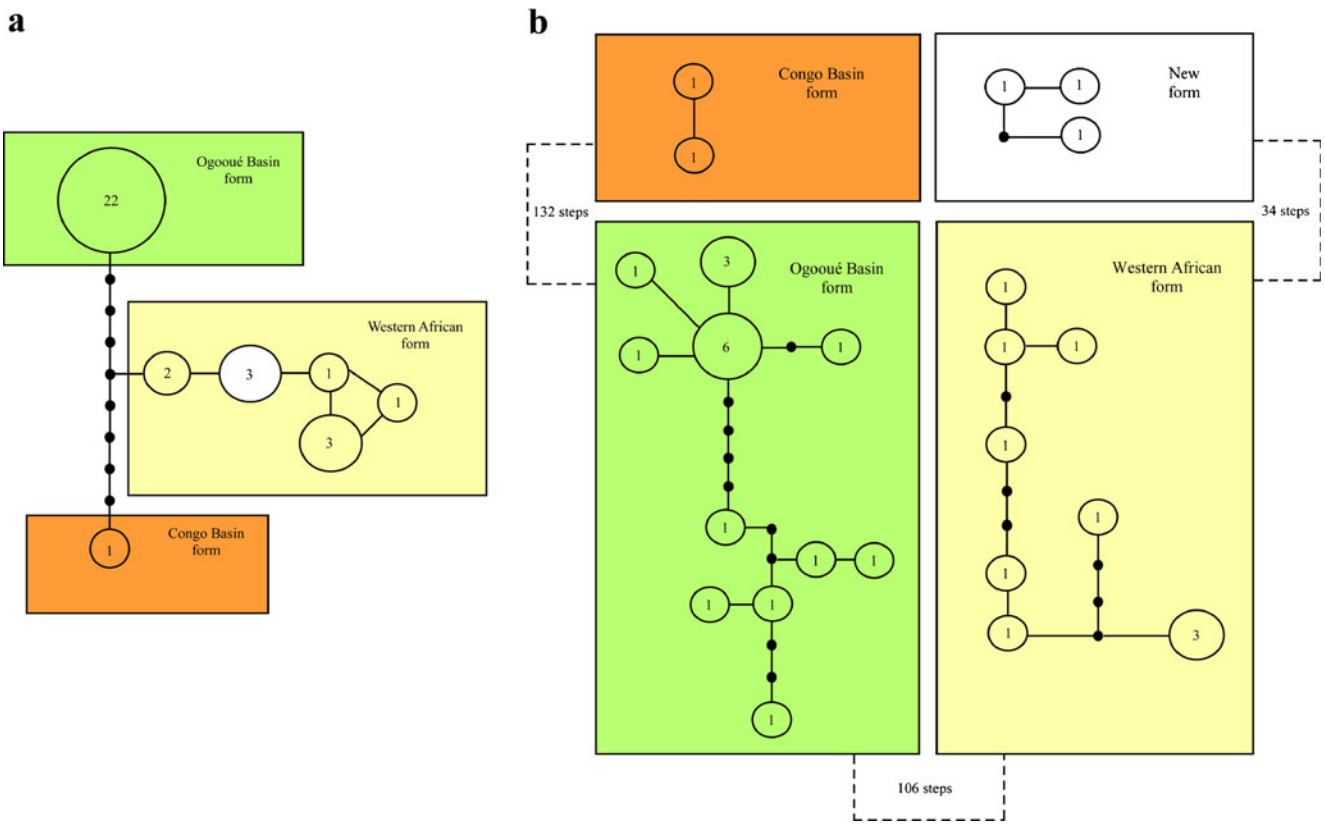


Fig. 3 Haplotype networks of nuclear RAG-1 and LDH-A gene sequences (**a**) and mitochondrial COI, cyt-b/CR and 12S genes (**b**) from 33 African dwarf crocodiles from zoological gardens in Europe. Numbers of individuals with identical sequences are indicated within each haplotype. For the nuclear network (**a**), the individuals from the fourth lineages in (**b**) are indicated as *white circles*. For the mitochondrial network (**b**), steps between networks are given in the image and are indicated with *dashed lines*

et al. 2003b; Hajibabaei et al. 2006, 2007; Ratnasingham and Hebert 2007) usually shows genetic distances of higher than 4 % between closely related chordate species (Hebert et al. 2003b; Eaton et al. 2009a). This corresponds to the genetic distances between the three different lineages (Congo Basin, Ogooué Basin, Western African form) of African dwarf crocodiles in zoological gardens showing values between 8.0 % and 11.0 % (COI). However, the genetic distance between the Western African form and the fourth clade with unknown origin is lower, 2.8 % based on COI. Other molecular studies show similar genetic distances in mitochondrial DNA between closely related crocodile species. For example, uncorrected distances within New World *Crocodylus* species are 1.1–4.6 % (Meredith et al. 2011) and 1.57 % between *Crocodylus porosus* and *Crocodylus siamensis* for mitochondrial genomes (Meganathan et al. 2010). Furthermore, Hekkala et al. (2011) found 3.2 % corrected genetic distances between "Eastern" Nile crocodile (*C. niloticus*) and New World Crocodiles and 3.1 % distances within the four New World species based on five mitochondrial genes. However, in most other cases the genetic distances between crocodile species are larger than 4 % (Hekkala et al. 2011; McAliley et al. 2006; Meganathan et al. 2010; Meredith et al. 2011). Thus, our evidence as to whether the new clade within *Osteolaemus* represents an evolutionary divergent lineage or is simply an artifact of a small samples size is inconclusive. Nevertheless, we favor the hypothesis of four *Osteolaemus* lineages (see below).

The analyses of haplotype networks based on the highly conservative nuclear gene fragments shows one haplotype network with three distinct groups (Fig. 3a). These groups are separated by a few steps and correspond to the three allopatric populations: the Congo Basin, Ogooué Basin and Western African forms (Eaton et al. 2009a). However, the nuclear sequences are highly conserved; the TCS default of a 95 % parsimony connection limit produced a connected network. In contrast, the analysis of the mitochondrial genes suggests four separate networks that do not share a connection. Thereby, consistent with the phylogenetic analyses of the combined dataset, the animals are grouped into four separate networks corresponding to the four clades described above (Fig. 3b).

Many studies have shown a correlation between the identity of species [often referred to as ESUs (evolutionary significant units)] and the 95 % connection limit of parsimony networks (Wiens and Penkrot 2002; Morando et al. 2003; Hart et al. 2006; Monaghan et al. 2006; Pons et al. 2006). The connection limit can be interpreted as an indicator for relationship borders in network analyses and DNA sequences of distinct species should be grouped into separate haplotype networks (Hart and Sunday 2007).

In summary, we detect a strong signal indicating the existence of a fourth *Osteolaemus* lineage in the investigated mitochondrial data. According to the phylogenetic species concept (PSC) sensu Mishler and Theriot (2000), species are the "least inclusive taxon" of a phylogenetic analysis. This concept refers to reciprocal monophyly, which should be found as well supported in phylogenetic analyses. Therefore, based on the PSC, we hypothesize the existence of four separate *Osteolaemus* species. With regard to the management of zoological collections, we suggest a conservative approach; our data support the recognition of four possible lineages within *Osteolaemus*. The datasets from Eaton et al. (2009a) and our study contain no samples from free-living wild animals yet that cluster within the new lineage. Therefore, animals from the fourth clade should be considered and treated as a separate lineage in zoological gardens. Otherwise, part of the biodiversity of *Osteolaemus* might be lost. For the future, it will be important to investigate more variable regions of the nuclear genome (e.g., microsatellites and AFLP markers) to confirm the existence of a fourth lineage. With additional nuclear markers and larger sample sizes, coalescence-based methods of species delimitation (e.g., SpeDeSTEM, Ence and Carstens 2010; or BPP, Zhang et al. 2011) could also be applied to test the validity of the fourth lineage.

Distribution and origin of *Osteolaemus* lineages in European zoos

Of the 75 dwarf crocodiles we sampled from European zoos, 12 crocodiles belong to the evolutionary lineage from Western Africa (*Osteolaemus* sp. nov.) and 42 animals are classified within the Ogooué Basin form (*Osteolaemus tetraspis)*. Only one zoo animal clusters with *Osteolaemus osborni*—a species that occurs in the Congo Basin. In our study, we also sequenced the DNA of a wild animal from Harcourt in Nigeria. This sequence clusters in the Ogooué Basin form. Two further samples from zoos in Mulhouse (France) and Bristol (UK), which are probably from Nigeria, also cluster within this evolutionary lineage, together with a museum sample supposedly coming from Nigeria (M6). Therefore, we suggest that the distribution of *Osteolaemus tetraspis* (Ogooué Basin form) is not only Gabon and Cameroon but also Nigeria (Fig. 1). Consequently, the Cameroonian highlands may not constitute a geographic barrier for dwarf crocodiles. At the moment, material from other regions of the species' range, e.g., Senegal, Uganda and North Angola, are still missing for molecular analyses. These representatives may help to clarify the complete phylogeny of the genus *Osteolaemus*. However, in some cases, data of provenance and places of discovery must be regarded as uncertain because of ambiguous or missing labels of museum samples. Some museum samples, which

clustered within the Ogooué Basin form, do not reveal the pattern of the distribution area of this form according to Eaton et al. (2009a). For example, M11 from Sierra Leone and M2 and M16 from Liberia, which constitute individuals from Western Africa, clustered within the Ogooué Basin form. According to the museum description, two samples (M5, M7) originate from the former French Congo, which ranged from Gabon to Central Africa (Democratic Republic of Congo and Central African Republic). The samples clustered within the Ogooué Basin form, which includes Gabon. However, the distribution pattern of the three major *Osteolaemus* lineages after Eaton et al. (2009a) is confirmed by several museum samples using the mitochondrial gene fragment COI (Supplementary material S9). According to studbook information, two dwarf crocodiles from a Spanish zoo (Z60, Z61) are native to Guinea, but according to the phylogenetic analyses they belong to different evolutionary lineages: the Ogooué Basin and Western African forms. Consequently, in this case Guinea might be an incorrect label for one of the animals. Furthermore, several states e.g., Guinea, Equatorial Guinea and Guinea-Bissau could be (mis)interpreted as "Guinea". These problems show the importance of comparative analyses between zoo animals and wild animals with known origin.

Consequences for the European studbook of *Osteolaemus*

Our studies suggest a new approach to the breeding management of dwarf crocodiles in European zoos. We found 15 potential hybrids between the Ogooué Basin and the Western African form. These are confirmed by the presence of heterozygous sites in informative nucleotide positions of nuclear genes, as well as by the fact that the parents are members of different lineages in our phylogenetic analyses. Consequently, there seems to be no reproductive barrier between those forms in zoological gardens. Therefore, the organization of breeding must be restructured and several specimens should be exchanged between European zoos for successful breeding within evolutionary lineages. Based on conservation and a prospective reintroduction in Africa, breeding of and with hybrids should be stopped and such zoo animals should not be reintroduced.

The majority of zoo animals belong to the Ogooué Basin form, with a few animals from Western Africa. In one zoo, a female of the Western African form and a male dwarf crocodile of the fourth evolutionary lineage have bred successfully. However, for further reintroduction programmes breeding should be restricted to animals within evolutionary lineages. The two Canadian zoo animals from Toronto are not registered in the European Studbook. But our studies suggest strongly that both belong to the Ogooué Basin form—*Osteolaemus tetraspis*.

In summary, we were able to group the investigated zoo animals into four different evolutionary lineages. This information will allow the European Studbook to make decisions about further breeding projects. Additional data of wild animals will be needed to clarify the taxonomy of all populations of African dwarf crocodiles. Particularly, individuals from other distribution areas lacking any data should be sampled to clarify the provenance of the fourth lineage. In addition, sequence data of further informative genes as well as microsatellite analyses can provide a better resolution of phylogenetic relationships and identification of hybridization events in future analyses. African dwarf crocodiles are threatened by habitat loss, hunting and trading in African countries (Eaton et al. 2009a; 2009b; Zoer 2010) and therefore listed in the Red list of IUCN and on Appendix I of CITES. The exact provenance of zoo animals is important for breeding, conservation, and future reintroduction, which will be successful only if these conditions are improved. Our study highlights the importance of the use of molecular data to distinguish between cryptic species for conservation and reintroduction programmes.

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