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Molecular identification of purebredness and kinship of the Philippine crocodile (*Crocodylus mindorensis*) and Cuban crocodile (*C. rhombifer*) for *ex situ* conservation management

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Annotation:

The current status of crocodilians recognizes them as a group under serious threat due to their habitat destruction and illegal poaching for their lucrative products. In addition to these threats, the elimination of spatial and temporal boundaries through modern anthropogenic pressures has facilitated hybridization in crocodiles by bringing together species that would otherwise not breed due to lack of opportunity. Here analyzed crocodiles, the Philippine crocodile (*Crocodylus mindorensis*) and the Cuban crocodile (*Crocodylus rhombifer*) are critically endangered and listed in CITES Appendix I.

This study deals with a significant portion of the Philippine and Cuban crocodile captive population in Europe based on mtDNA, nucDNA and microsatellites. The species genetical purity of 13 specimen of *C. mindorensis* was determined on the ground of testing maternally inherited mitochondrial gene cytochrome *b* and and D-loop loci from the mitochondrial control region as well as two nuclear markers, LDHA and C-*mos.* Also the purity of 11 out of mentioned 13 individuals on the basis of several (7) microsatellite loci, while possibly confirming a supposed hybrid origin of two crocodiles with mixed morphotype. Then a purity of 4 samples of the Cuban crocodile was verified with cyt *b* gene and nuclear DNA purity of all 7 tested individuals with LDHA gene and 13 microsatellites.

Based on the obtained genetic characters I proceeded a likely kinship of the two groups of crocodiles and subsequently suggested an optimal breeding management within the *ex-situ* conservation.

Contents

1. INTRODUCTION

Crocodilians present an ancient lineage of archosaurian reptiles comprising 9 genera and 23 species, including alligators, caimans, crocodiles, false crocodiles, false gharial and gavials (Miles et al., 2009). Currently there are three families recognized within the Crocodylia - Alligatoridae (*Alligator, Caiman, Melanosuchus* and *Paleosuchus*), Crocodylidae (*Crocodylus, Osteolaemus* and *Tomistoma*, according to some studies) and Gavialidae (including only one species, *G. gangeticus*, or two including *Tomistoma* as in other studies) (Zhang et al., 2011; Willis et al., 2007). Formerly, the genus *Crocodylus* was known to consist of 12 species including *Crocodylus cataphractus*, but recent studies have provided consistent evidence for this species as a non-*Crocodylus* member (Brochu, 2000; McAliley et al., 2006) and thus the name *Mecistops cataphractus* was resurrected.

On the other hand, de Smet (1999) stated that in *Crocodilus niloticus*, there have been distinguished different forms, tropical and Saharan, which should become a subject for a profound study of the genetic and morphological variation before any reintroduction occurs. Recent studies (Schmitz et al., 2003; Oaks, 2011; Hekkala et al., 2011; Meredith et al., 2011) have confirmed this and found two distinct clades representing non-sister species in *C. niloticus*. Even a taxonomic revision was suggested as well as resurrection of *Crocodylus suchus* Geoffroy, 1807. Therefore the number of members of the genus *Crocodylus* will probably change again soon. Moreover, Shirley et al. (2013) established that *Mecistops cataphractus* actually consists of two distinct species, the West African and the Central African. These findings suggests that the crocodilian phylogeny may not be clear yet and that it is possible that some cryptic species are still to be discovered.

Despite their long and rich fossil record and impressive former and recent diversity, many crocodilians have declined rapidly due to multifactorial human destructive activities during several last centuries. The current status of the crocodilians recognizes them as a seriously endangered group due to habitat destruction and illegal poaching for their lucrative products (Santiapillai and de Silva, 2001; Stuart et al., 2002). As all crocodilians are under varying degrees of threat because of their overexploitation, these species have been listed in Appendix I (17 species) or II of CITES; and considering the IUCN Red list,

3 species are listed as vulnerable, 1 as endangered and 6 as critically endangered (http://www.iucnredlist.org).

However, illegal trade continues to date and exacerbates their survival (Miles et al., 2009). These exploitations have impacted crocodilian numbers and thus inevitably the genetic structure and diversity within their populations (Davis et al., 2002). In addition to the previous threats, the elimination of spatial and temporal boundaries through modern anthropogenic pressures has facilitated hybridization by bringing together crocodilian species that would otherwise not breed due to lack of opportunities (Fitzsimmons et al., 2002).

Such problems exemplify the need for further polymorphic markers to assist in population studies to assess the vulnerability status of some species. Genetic studies provide information pertinent to the development of management plans by identifying conservation and breeding units for many threatened and endangered species (Meganathan et al., 2009; Miles et al., 2009; Verma and Singh, 2003; Hsieh et al., 2001).

Crocodiles analyzed in this thesis, i.e. the Philippine crocodile (*Crocodylus mindorensis*) and the Cuban crocodile (*Crocodylus rhombifer*), are criticaly endangered (IUCN Red list: Crocodile Specialist Group 1996; Targarona et al., 1996) and are both listed in Appendix I of the Washington Convention known as CITES.

1.1. *Crocodylus mindorensis* (Philippine crocodile)

The Philippine crocodile has long been treated as *C. novaeguineae mindorensis*, a sub-species of the New Guinea crocodile, until Hall (1989) provided an evidence of the distinctiveness of the Philippine crocodile. Nowadays *C. mindorensis* is generally considered to be a separate endemic species of the Philippines. It was historically widely distributed throughout the archipelago and probably occurred on all larger Philippine islands. The probable exception is the Palawan island which exhibits more faunal affinities to Borneo than to the other Philippine islands.

The Philippine crocodile is a relatively small freshwater crocodile that lives in rivers, creeks, ponds and marshes from sea level up to at least 850 m a. s. l. in the Cordillera

Mountains of Luzon (Manalo, 2008). It has also been observed in saline waters along the coast of Luzon where it moves between small creeks through the sea (Van Weerd, 2010). In several areas, *C. mindorensis* and *C. porosus* appear to occur sympatrically. This is the case in Ligawasan Marsh on Mindanao (Pomares et al., 2008) and in the coastal wetlands of Isabela on Luzon (Fig.1, though the Isabela province is not marked there). Such sympatrical occurrence probably allow emerging of hybrid zones.

The IUCN Red List treats this species as critically endangered based on the following criteria: observed decline in extent of occurrence greater than 80% in 3 generations, less than 250 adults persisting in the wild and highly fragmented and declining populations. The CSG (Crocodile Specialist Group) Action Plan establishes the availability of recent survey data as adequate and suggests the highest need for population recovery whilst the potential for sustainable management is considered low. Despite this view, the *in-situ* conservation of Philippine crocodiles started seriously only in 1999 after the discovery of a remnant *C. mindorensis* population in Isabela Province.

Limited information is available on the Philippine crocodile regarding the levels of genetic diversity either relative to the other crocodilian species or among populations of the species itself. With only two remaining areas with extant populations, potentially low levels of genetic diversity are a conservation concern (Hinlo et al., 2014). The extant populations from both of these distantly isolated areas show less genetic diversity than what has been detected in other crocodilian species in previous studies. These populations have low effective population sizes relative to other studied species. There is no indication of selection being a differentiating factor but the distance and isolation would be expected to drive the genetic drift (Hinlo et al., 2014). Slightly elevated relatedness estimates suggest that future generations within populations from both areas could face unavoidable mating of related individuals and the potential consequences of inbreeding. A genetic augmentation should be considered to offset these potential problems, whether by reintroduction from captive populations or by translocation between the populations.

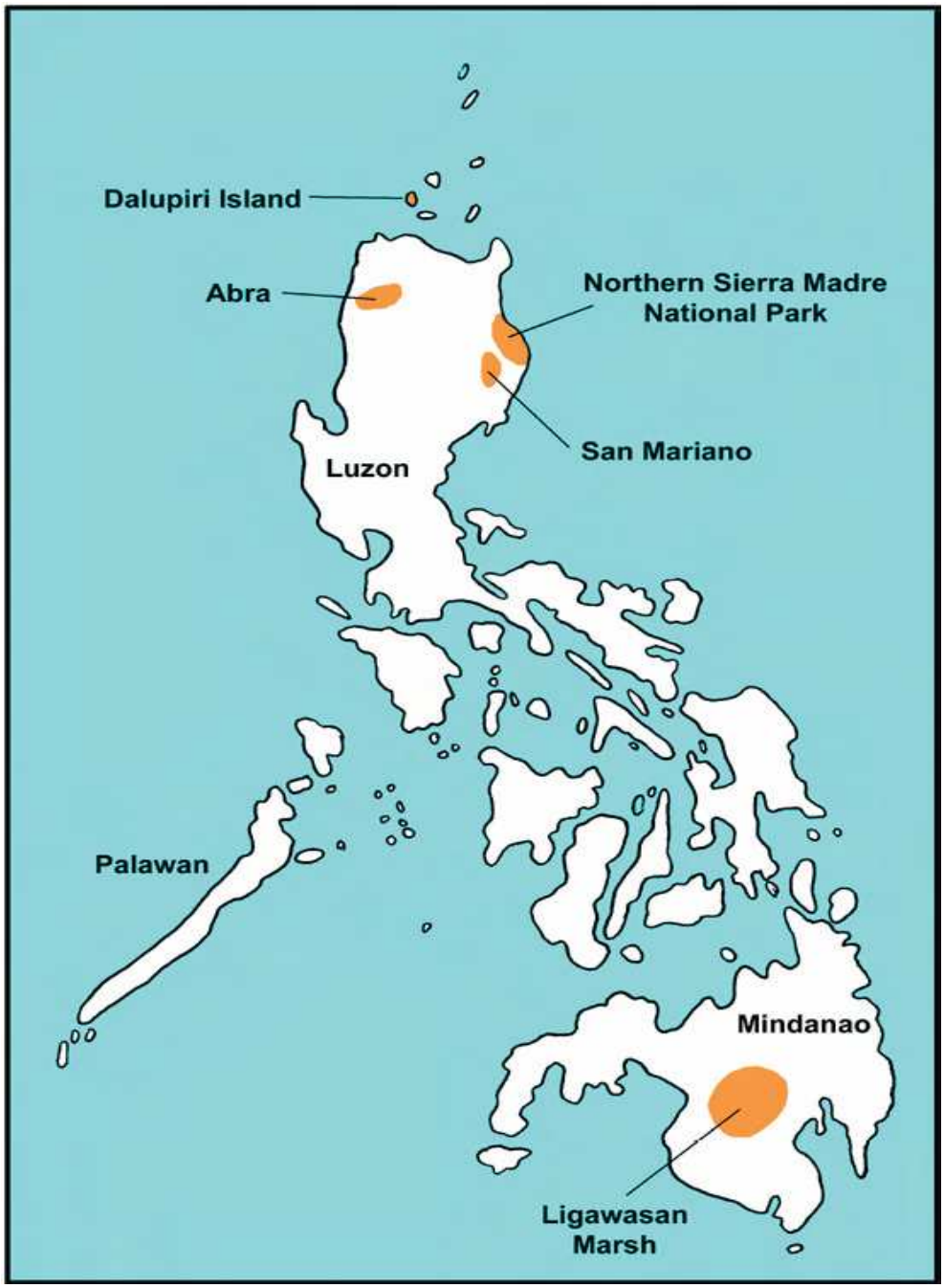

Fig. 1. The likely recent distribution of *Crocodylus mindorensis* (from Van Weerd, 2010).

1.2. *Crocodylus rhombifer* (Cuban crocodile)

Crocodylus rhombifer is primarily a freshwater species, although there are some historical reports about its presence in brackish water areas along the Bay of Pigs (Gundlach, 1880). The Cuban crocodile is considered to be the most morphologically, ecologically, and behaviorally distinct taxon among all *Crocodylus* species. Thorbjarnarson et al. (2008)

associated these differences with an adaptive evolution in Cuba and adjacent Caribbean islands, where its ancestor became a terrestrial or semi-terrestrial predator during the Pleistocene. *C. rhombifer* is naturally sympatric with the American crocodile (*C. acutus*) in some areas of the Zapata Swamp, and natural hybridization zone has been detected based on present morphotypes. Yet *C. acutus* has a more extensive distribution (Weaver et al., 2008).

C. rhombifer and *C. acutus* were subjected to extensive hunting pressure from the middle of the 19th century through to the 1960´s, resulting in drastic population declines of both species (Rodríguez-Soberón et al., 2000). Although the *C. acutus* population has recovered and is now distributed in most of coastal areas of Cuba, *C. rhombifer* remains rare, consisting only of 3000 individuals, including 1000 females (Ramos-Targarona et al., 1994; Ramos-Targarona, 2000; Rodríguez-Soberón et al., 2000).

Although *C. rhombifer* is currently restricted mainly to the Island of Cuba (Fig. 2), fossil records from the Grand Cayman Islands and the Bahamas indicate that this species was much more widespread during the Pleistocene (Varona, 1966; 1986; Morgan et al., 1993; Franz et al., 1995; Steadman et al., 2007).

C. rhombifer has been assessed as Critically Endangered based on a decline greater than 80% in its population over the last three generations due to the decline in habitat quality, exploitation, limited distribution, hybridization and the introduction of exotic animals into their environment (Ross, 1998). Illicit hunting of crocodiles for meat has rapidly increased the population decline (Targarona et al., 1996).

Hybridization was reported to have occurred between captive populations of *C. rhombifer* and *C. acutus* in breeding pens of the Laguna del Tesoro farm in Cuba (Ross, 1998) as well as in the wild (Ramos-Targarona et al., 1994). All hybrid individuals had *C. rhombifer*-like mtDNA, which suggests that hybridization in captivity occurs typically between a female *C. rhombifer* and a male *C. acutus* (Weaver et al., 2008). Milián-García et al. (2011) proved that hybridization between these two species has been a historical as well as a current phenomenon, when they confirmed the findings of Weaver et al. (2008), that there are two mtDNA haplotypes in *C. rhombifer* in Cuba, α and β, whereas only α do not show any hybridization or introgression with *C. acutus.*

Fig. 2. Map showing the recent distribution of *Crocodylus rhombifer* (from Targarona et al., 2012)

1.3. Hybridization

Natural hybridization can be part of natural evolutionary processes; however, the increase of anthropogenically mediated hybridization has been implicated in the extinction of many taxa (species, subspecies, or locally adapted populations) (Milián-García et al., 2011). Hybridization is also a serious conservation concern as it is often undetected, especially if hybrids are morphologically hardly distinguishable, such as in the case of *C. rhombifer* and *C. acutus* (Rhymer and Simberloff, 1996; Allendorf et al., 2001; Fitzsimmons et al., 2002; Allendorf and Luikart, 2007). Recognized introgression of the mtDNA from *C. rhombifer* into *C. acutus* could also explain some incongruences between mtDNA and morphological results (Milián-García et al., 2011).

Hybridization with introgression has been reported in other animal species as the mechanism of speciation (Ferris et al., 1983; Tegelström, 1987; Hird and Sullivan, 2009; Larsen et al., 2010). Moreover, hybridization between *Crocodylus* species is quite common, both in the wild and in captivity (Fitzsimmons et al., 2002; Hekkala, 2004; Ray et al., 2004; Russello et al., 2006; Rodríguez et al., 2008; Weaver et al., 2008).

Yet hybridization plays a dual role in evolutionary biology and conservation. Evolutionarily it may contribute to the genetic variability and increase fitness in small populations (partially due to emerging of beneficial local adaptations) or it may result in melting of two previously distinct evolutionary lineages (Grant and Grant, 1992; Clarke et al., 1998; Zimmer, 2002; Coyne and Orr, 2004). On the other hand, hybridization can also result in decreased fitness of hybrids (Milián-García et al., 2011).

Captive breeding can moreover force unnatural interspecific hybridization in mixed-species exhibitions or enclosures due to the lack of opportunity to mate with an optimal sexual partner (for review in mammals see Groves and Robovský, 2011 and Fitzsimmons et al., 2002 in crocodiles). Unfortunately, interspecies hybridizations could occur as well in the wild where they are associated with the elimination of spatial and temporal boundaries through modern anthropogenic pressures (Fitzsimmons et al., 2002). Anthropogenic hybridization may be intentional, particularly for captive, commercially used species and/or it may result from the introduction of exotic species and habitat fragmentation and/or alternation (Allendorf et al., 2001). However, more serious conservation concern is the unintentional hybridization.

The ability to detect hybrids is essential in selection of specific individuals suitable for reintroductions into extirpated areas (Allendorf et al., 2001; Fitzsimmons et al., 2002). Hybrid introgression has been detected in some New World crocodilians (Hekkala, 2004; Ray et al., 2004; Rodríguez, 2007; Cedeño-Vázquez et al., 2008; Rodríguez et al., 2008) and considering *C. rhombifer*'s smaller population numbers and its frequent sympatry with larger *C. acutus*, the genetic integrity of this species is at risk (Weaver et al., 2008).

It has been suggested that detecting hybrids is less exhaustive when the two parental crocodiles possess different karyotypes, but detection of hybridization between individuals with similar karyotypes requires more in-depth analysis (Chavananikul et al., 1994; Fitzimmons et al., 2002). Considering the chromosomal and biochemical similarity (Cohen and Gans, 1970; Densmore, 1983) and a relatively recent divergence (Brochu, 2000) between *C. rhombifer* and *C. acutus*, detection of their hybrids based on morphological characters alone may be very problematic. In this case, the use of molecular markers is highly warranted. Moreover, molecular markers are much easily analyzable and sensitive

than karyotype and they are also suitable from the tissue samples requirement´s point of view.

Recent systematics studies identified hybrids between *C. mindorensis* and *C. porosus* at Palawan Wildlife Rescue and Conservation Center (from the analyses of both mitochondrial DNA (D-loop and ND4) and nuclear DNA (C-*mos*) gene sequences) (Louis and Brenneman, 2007; Tabora et al., 2012). These studies validated previous concerns regarding reintroduction candidate purity, thus warranting forensic diagnoses prior to release.

1.4. Molecular markers

The application of genetics in conservation efforts has increased dramatically over the past decades. Molecular genetic methodology has been used to address taxonomic issues, assess genetic variability and inbreeding, track gene flow and detect hybridization, all in an effort to conserve genetically healthy populations and aid in the identification of evolutionary significant units (Fleischer, 1998). The use of nuclear DNA (nucDNA) and mitochondrial DNA (mtDNA) sequence data in crocodilian research has increased our understanding of the genetic variability (Flint et al., 2000; Ray et al., 2004; Russello et al., 2007), hybridization (Fitzsimmons et al., 2002; Ray et al., 2004; Cedeño-Vásquez et al., 2008), differences between individuals (Farias et al., 2004), populations (Vasconcelos et al., 2006; 2008) and species (Li et al., 2007; Gatesy and Amato, 2008; Meganathan et al., 2009; Meganathan et al., 2010) for wild and/or captive individuals or populations.

Molecular and phylogenetic approaches might overcome all factors complicating conservation of crocodiles, because they are able to determine the species status of captive individuals, detect the hybrid origin of some of them and also specify intraspecific kinship relationships (Allendorf et al., 2001; Fitzsimmons et al., 2002). Molecular genetic markers can facilitate the identification of parental vs. hybrid individuals in wild and captive populations as well as characterize the population structure, allowing wildlife managers to assign unknown individuals to their geographical source population (Milián-García et al., 2011). Characterizing intraspecific genetic variation also helps captive breeding programs to avoid out-crossing of divergent lineages (Densmore and Ray, 2001; MacGregor, 2002) and improve the efficiency of reintroduction programs (Densmore and Ray, 2001;

Venegas-Anaya, 2001; Venegas-Anaya et al., 2008). Effective and long-term conservation of the crocodilians will, therefore, benefit significantly from the identification of genetically pure and hybrid populations.

Maternaly inherited DNA (mtDNA) has been routinely applied on the detection of crocodile phylogenetic relationships (Fitzsimmons et al., 2000; Dessauer et al., 2002) and the purity of studied individuals from the maternal side (Dever et al., 2002). MtDNA genes (i.e. cytochrome *b*) and loci (i.e. mitochondrial control region with the D-loop) are available for the majority of crocodile species (Ray and Densmore, 2002). The effectiveness of cytochrome *b* (Cyt *b*) gene as a species-specific marker (Hsieh et al., 2001; Bravi et al., 2004; An et al., 2006; Caine et al., 2006) and as a significant tool for evolutionary studies (Irwin et al., 1991; Su et al., 1999; Gatesy et al., 2004) has already been established.

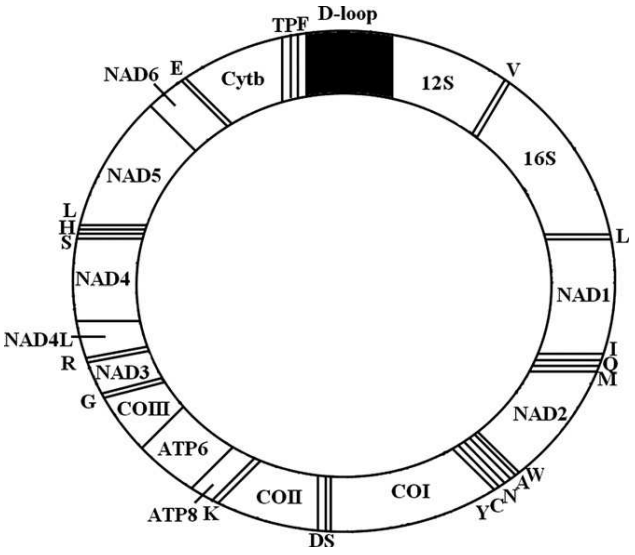

Fig 3. Complete mitochondrial genome organization of crocodiles. The tRNAs are identified by the single-letter amino acid code (from Zhang et al., 2011)

Microsatellites are very sensitive and useful genetic nuclear DNA markers for genome mapping due to their hyper-variability and abundance throughout most vertebrate genomes (Toth et al., 2000). Typing of the microsatellite DNA loci by routine polymerase chain reaction (PCR) was developed almost 20 years ago (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989) and has since facilitated the construction of dense genetic maps in many species (Miles et al., 2009). Today, PCR is performed with

a fluorescent dye-labeled primers and detection is carried out on an automated capillary electrophoresis-based DNA sequencer.

As one of the most promising and informative markers, microsatellites have been used in variety of studies to assign parentage within a family (Glenn et al., 1996; Isberg et al., 2004) and to estimate relatedness in cases when pedigree relationships are unknown (Isberg et al., 2006). Microsatellite DNA loci serve as the dominant genetic tool for addressing questions associated with genetic diversity in many wildlife species, including crocodilians, where microsatellites have been used to assess not only the genetic diversity, but also the mating behaviour and hybridization as well as dispersal systems in a variety of species (Glenn et al., 1996; 1998; Fitzsimmons et al., 2002; Dever et al., 2002; Davis et al., 2002; Dessauer et al., 2002; Verdade et al., 2002; Isberg et al., 2004; 2006). Despite these facts, informative microsatellite markers still do not exist for many of the crocodilian species (Glenn et al., 1998).

To date, most of the microsatellites cited in the literature were originally developed from either the American alligator (*Alligator mississipiensis*) or the saltwater crocodile (*Crocodylus porosus*) and later cross-amplified in other closely related non-target species for a wider application. Glenn et al. (1996) noted that microsatellites isolated from the American alligator were significantly less likely to amplify orthologous loci from a distantly related species such as those in the crocodile family. This is not surprising given the divergence time of alligators and crocodiles, which is estimated to be 140 MYA (Janke et al., 2005). Thus, a major limiting factor currently affecting the broader application of microsatellites in crocodilian research, especially for the ‘‘true crocodiles’’, is the lack of suitable primers capable of amplifying homologous loci in a large range of species (Miles et al., 2009).

Molecular markers have been used routinely to characterize the threatened species and populations (Frankham et al., 2002) but genetic studies first require a point of reference to accurately assess the species assignments. Developing a pure-breeding stock of *C. rhombifer* will be essential in maintaining the genetic integrity of the species, which is why any potential hybridization with other species can be a problem in captive populations (Weaver et al., 2008).

Comprehensive genetic testing identifies hybrids in the collection that can be separated out of the gene pool before a hybrid swarm is created that could have a detrimental effect on the conservation management of the species (Allendorf et al., 2001). The removal of the suspected hybrids could protect the genetic integrity of the species, especially if used as reintroduction candidates or to augment the genetic diversity of the wild populations (Rhymer and Simberloff, 1996).

1.5. *Ex-situ* conservation

Combination of the *ex situ* (zoos and breeding farms) and i*n situ* conservation approaches seems to be the most effective tool for the conservation of the present crocodilian diversity (see Crocodiles Status Survey and Conservation Action Plan, 2010, IUCN SSC Crocodile Specialist Group). The captive conservation management as Species reservoir or Noah´s ark proves to be necessary for species with limited suitable habitat in the wild (i.e. Philippine crocodile).

Captive breeding should be ideally based on many purebred individuals that should be mixed in the combinations that preserve the genetic variability as much as possible in the long-term horizon (Frankham et al., 2003; Allendorf and Luikart, 2007; Witzenberger and Hochkirch, 2011). Under these conditions, every additional unrelated founder specimen has a great importance for the captive conservation management in order to maintain the genetic diversity (Frankham et al., 2003). On the contrary, unknown origin of some kept animals or cross-breeding crocodiles in groups with a limited data about parentities are negative factors in the optimal conservation management.

2. STUDY OBJECTIVES

This study deals with a significant portion of the Philippine and Cuban crocodile captive population in Europe based on mtDNA, nucDNA and microsatellites. According to the ISIS (International Species Information System) database, 54 Cuban crocodiles are kept by 24 breeders wordlwide (in Europe 21 individuals with 11 breeders) and 31 Philippine crocodiles worldwide (10 breeders, in Europe 7 individuals with 3 breeders). Yet the genetic purity of some of them is not certain, especially in *C. rhombifer* (Weaver at al., 2008).

These numbers do not include individuals kept in the „Crocodile ZOO Protivín“ which is not an EAZA member (although the membership in EAZA does not necessarily relate with being a part of the ISIS database) and keep 45 Cuban crocodiles and 13 Philippine ones. This institution is very successful in breeding several crocodile species and this study is used for establishing new breeding groups in both critically endangered crocodiles based on molecular evidence. Identification of the purebred individuals could dramatically improve the European and worldwide breeding of *C. mindorensis* and *C. rhombifer*.

The aims of this study are 1) to determine the purity and kinship of 13 Philippine crocodiles and 2) to determine the purity of 7 Cuban crocodiles; both kept in the Crocodile ZOO Protivín; and 3) to try to establish a breeding management of these species based on the scientific evidence.

3. MATERIAL AND METHODS

3.1. Samples

Altogether we obtained samples from 20 individuals (see Table 1). In 17 cases blood samples were taken (13 *C. mindorensis* and 4 *C. rhombifer*) and in 3 remainig animals we performed the mouth swabs (all *C. rhombifer*), where the crocodiles chewed on a piece of gauze. Blood samples were collected via the caudal sinus during other contemporaneous veterinary procedurs and stored in 96% ethanol at -20°C prior to isolation.

DNA was extracted using JET QUICK Tissue DNA Spin Kit (Genomed) or Genomic DNA Mini Kit (Geneaid), then electrophoresed on a 1% agarose gel and also flourometricaly measured. In case of the mouth swabs, DNA was extracted using 5% Chelex and Proteinase K (following standard protocol used in forensics to extract DNA from saliva on fabric). But because the yield of DNA was insufficient, it was necessary to repeat this once again with more intense extracting process.

Most of the Philippine crocodiles were transported to the Crocodile ZOO Protivín from the Avilon ZOO in Manila. According to this facility, individuals I, J and K possibly come from the same clutch and are supposed to be offspring from M, D or E, respectively. But the manager and operator of the Crocodile ZOO Protivín considers that information quite unreliable. The „hybrid phenotype“ in individuals G and H (Tab. 1) was determined by the manager of the Crocodile ZOO Protivín based on the pattern of the ventral scales.

With the Cuban crocodiles the situation is much clearer. Individuals R_0, R_1, R_2 and R_3 are siblings and also offspring of R_4 and R_5. Hence the specimen R_6 is the only one unrelated to the other individuals among tested *C. rhombifer*. Such known relatedness could be theoretically used as a possible control factor for determining the effectivness of using pedigree relationships of the analysing programme ML Relate (see Results and Discussion) to establishing genetic variability between animals.

Individual code	Species	ID chip	Notes
A	*C.mindorensis*	985120029025105	male "Monty"
B	*C.mindorensis*	985120027838974	female "Golda"
C	*C.mindorensis*	956000002283302	male "Karel"
D	*C.mindorensis*	985120024073321	female "Světlana"
E	*C.mindorensis*	985120029043711	female "Minda"
F	*C.mindorensis*	985120028000000	female
G	*C.mindorensis*	00064D31BF	male, hybrid phenotype
H	*C.mindorensis*	00064DF616	female, hybrid phenotype
I	*C.mindorensis*	956000002314019	female "Malý Jack"
J	*C.mindorensis*	956000002289357	unknown "Malá Světlana"
K	*C.mindorensis*	956000002275518	male "Malá Minda"
L	*C.mindorensis*	956000002277358	female "Ocásek"
M	*C.mindorensis*	956000002339585	male "Jack"
$R=R_0$	*C.rhombifer*	/	female
R_1	*C.rhombifer*	900032000380661	unknown
R_2	*C.rhombifer*	900032000380663	female
R_3	*C.rhombifer*	900032000380694	male?
R_4	*C.rhombifer*	/	female "Lady"
R_5	*C.rhombifer*	/	male "Pirát"
R_6	*C.rhombifer*	/	female "Kačenka"

Tab. 1. Overview of the crocodiles analysed in this thesis with some additional information.

3.2. Mitochondrial DNA

These individuals were analysed for mitochondrial cytochrome *b* gene using primers crCYTBfor and crCYTBrev (Weaver at al., 2008) and D-loop using primers t-PHE-L and CR2H (Ray and Densmore, 2002). The cyt *b* gene was amplified and analysed for both of the studied species, as the purity of *C. rhombifer* individuals was not questionable and the aim was just to confirm this purebredness. Aside that we wanted to find out with what haplotype group (α or β) these animals belong. Whereas the genetic purity of *C. mindorensis* was disputable and so two mtDNA genes were analysed to obtain more certain conclusion.

PCR were performed in a total volume of 25µl, using 12,5µl Combi PPP Master Mix, 1,5µl 5´primer, 1,5µl 3´primer, 7µl PCR H_20 and 2,5µl DNA. Amplification was run on XP Cycler (Bioer Technology). Thermocycling conditions for cytochrome *b* consisted of an initial denaturation step of 2 min at 94°C, then 33 cycles of 30 sec at 94°C, 1 min at 58°C, and 45 sec at 72°C; with a final extension of 7 min at 72°C. Because of a problematic amplification of a few samples a slight modification was made; as follows: the denaturation step 2 min at 95°C, then 38 cycles of 15 sec at 95°C, 30 sec at 55°C and 1 min at 72°C. The final extension was set to 10 min at 72°C. In some individuals it was necessary to perform the PCR reacion repeatedly, yet different times for diferent samples (the same is true for the step of the DNA extraction; where PRC amplification was repeatedly unsuccessful, the DNA extraction was repeated).

The thermocycling conditions for D-loop gene followed Hauswaldt et al. (2013) and consisted also of a initial denaturation step of 2 min at 94°C, then 35 cycles of 120 sec at 94°C, 45 sec annealing at 60°C and 80 sec extension step at 72°C and final extension of 5 min at 72°C.

The amplified samples were electrpphoresed in a 1% agarose gel to verify PCR products. Unincorporated dinucleotides and primers were then removed from these PCR products using the JET QUICK PCR Purification SPIN KIT or via combination of enzymes EXO I. (exonuclease I.) and CIAP (Alkaline Phosphatase), while adding 0,2µl of each into the PCR product and cleaned in a cycler for 15 min at 37°C and another 15 min at 80°C. 4µl of obtained PCR product for each individual was subsequently mixed with 3µl of PCR H_20 and 0,5µl of primer, making a total volume of 7,5µl, that was used for DNA-sequencing.

3.3. Nuclear markers

We also used two neclear genes to distinguish the purity of our samples. Namely we chose C-*mos* protooncogene using primers CmosF and CmosR designed by Meganathan et al. (2010) and lactate dehydrogenase A gene (LDHA) using primer pair LDHA17-F/LDHA17-R1 (Gatesy et al., 2004). As mentioned in mtDNA markers, also here only one of the two analysed nuclear genes was carried out for both of the studied species

(i.e. LDHA), because of a greater need of ascertaining of the purebredness in *C. mindorensis* and ensuring of non-hybrid origin in all individuals of the Philippine crocodile.

PCR thermocycling conditions in both primer pairs started with an initial denaturation step of 2 min at 94°C, then for C-*mos* consisted of 38 cycles of 60 sec at 94°C, 45 sec at 58°C, and 80 sec at 72°C; with a final extension of 8 min at 72°C. For the LDHA there were 45 cycles of 45 sec at 94°C, 45 sec at 53°C, and 60 sec at 72°C; the final extension at 72°C lasted 6 min. After the PCR followed the electrophoresis and removing of the unincorporated dinucleotides and primers as mentioned with the mitochondrial DNA.

3.4. Microsatellites

To identify the kinship and purity, 7 microsatellite loci were used (C391, CU5 123, CUD 78, Cj18, CUJ 131, CR52, CUI 99.2; designed by Fitzsimmons et al., 2000) for studied individuals of *C. mindorensis* and 14 loci (Cj16, Cj18, Cj35, Cj101, Cj104, Cj109, Cj119, Cj127, Cj128, Cj131, Cp10, C391, CU5 123, CUJ 131; designed by Fitzsimmons et al. 2000, plus primer for locus Cj109 taken from Dever and Densmore, 2001) for studied individuals of *C. rohombifer*. One primer of each pair was always labeled with a fluorescent dye; FAM, NED, PET, or VIC. Again, in some individuals or with some of the primers it was necessary to perform the PCR reaction repeatedly, yet different times for diferent samples.

Crocodylus mindorensis:

Microsatellite loci were amplified in two multiplex sets (set 1: C391, CU5 123, CUD 78, CJ18; set 2: CUJ 131, CR52, CUI 99.2). PCR reactions were performed in a total volume of 25µl consisting of 1µl of each primer, 12,5µl Combi PPP Master Mix, 2,5µl PCR H_20 and 2µl DNA. Amplification was run on XP Cycler (Bioer Technology) using the same PCR profile for all combination of primers. This profile contained an initial denaturation step of 2 min at 94°C, then 33 cycles of 30 sec at 94°C, 30 sec at 58°C, and 45 sec at 72°C; with a final extension of 5 min at 72°C. 1 µl of PCR products was mixed with 12 µl deionized formamide and 0,3 µl GeneScan Internal Lane Size Standard-GeneScan-500 [TAMRA] (Applied Biosystems).

Crocodylus rhombifer:

Primers for 14 loci were divided into three multiplex sets, based on fluorescent label colour, base pair range and annealing temperature (set 1: Cj16, Cj127, C391, CU5-123, CUJ131; set 2: Cj18, Cj104, Cp10 and set 3: Cj35, Cj101, Cj109, Cj119, Cj128, Cj131). PCR reactions were performed in a total volume of 25 μl which always contained 12,5 μl Combi PPP Master Mix and 2 μl of DNA. The volume of each primer was 0,5 μl, except for Cj127, C391 (set 1), Cj18 and Cj104 (set 2), where the content had to be reduced to 0,3 μl. PCR H_20 was then added to comprise the final volume.

Amplification was run on XP Cycler (Bioer Technology) using a universal PCR profile differing only in the annealing temperature for each set. This profile contained an initial denaturation step of 5 min at 94°C, then 35 cycles of 45 sec at 94°C, 45 sec at 60°C (set 1)/ 52°C (set 2)/ 54°C (set 3), and 1 min at 72°C; with a final extension of 10 min at 72°C. The annealing temperature for locus Cj101 had to be changed to 50°C to optimize its amplification.

Same as already mentioned above, 1 μl of PCR products was then mixed with 12 μl deionized formamide and 0.3 μl GeneScan Internal Lane Size Standard-GeneScan-500 [TAMRA] (Applied Biosystems).

3.5. Data analysis

Phylogenetic analysis of cytochrome *b*, D-loop, LDHA and C-*mos*:

Obtained sequences were assembled using the programme Seqman (DNASTAR 2001) and then compared with all available sequences for particular genetic markers from the GenBank database (used sequences are included in the phylogenetic trees, see Results). They were aligned using automatic strategy in the Multiple alignment programme MAFFT version 7 (Katoh and Standley, 2013). All data were analyzed by two approaches, MP (maximum parsimony) (resulting trees in Appendix) and BI (Bayesian inference). The unweighted maximum-parsimony analysis was applied to the matrix (NONA ver. 2.0; Goloboff, 1999) heuristics, option hold10000 mult*1000 hold/10 (for cyt *b* and D-loop) or hold1000000 mult*10000 hold/100 (for other markers with less number of sequences), unconstrained search strategy with TBR branch swapping; Winclada, ver. 1.00.08; Nixon,

1999). For all gained strict consensus we noted the lenght of branches (L), retention index (RI) and consistent index (CI).

As previously mentioned, we also employed a Bayesian inference method (BI) for separate phylogenetic analyses of all 4 genes. The best-fitting substitution model of DNA sequence evolution was selected by jModelTest 0.1.1 (Posada, 2008) under the Akaike Information Criterion (AIC). Different model was determined for each dataset (e.g. GTR+I+G for cyt *b*; GTR+G for D-loop; GTR+G for LDHA and HKY+G for C-*mos*). Bayesian phylogenetic analysis was conducted with a Metropolis-coupled Markov chain Monte Carlo algorithm (Altekar et al., 2004) as implemented in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The nucleotide data were run for 5.000.000 generations with two runs and four chains for each run. Sampling frequency of every 100th generation produced 50.000 sampled trees. First 20% of trees (10.000) was discarded as a burn-in. We consider probabilities (pp) of 95% or greater to be significantly supported (cf. Schmitz et al., 2003).

I also used the Bioedit (Hall, 1999) to visualise variable sites for the nuclear genes C-*mos* and LHDA between potentially hybridizing species (*C. novoguineae* and *C. porosus* for *Crocodylus mindorensis* and *C. acutus* for *Crocodylus rhombifer*)

Microsatellite analysis:

Fragment analysis was carried out on an ABI 3130 Genetic Analyser (Applied Biosystems) and electrophoretograms were then manually analysed in GeneMapper v.3.7 (Applied Biosystems). ML-Relate, a computer programme, was used for maximum likelihood estimation of relatedness and relationship (Kalinowski et al., 2006). This program is useful for discriminating among four pedigree relationships: unrelated (U), half-siblings (HS), fullsiblings (FS), and parent-offspring (PO). The programme assesses these relationships from the maximum likelihood relatedness based on the coefficient of relatedness (r).

4. RESULTS

Although the fluorimetrical measurement showed a minimal content of DNA in the isolates from the mouth swabs, even after several attempts I was unable to get any functional PCR product for the cytochrome *b* gene. The same is valid for the LDHA gene (actually only 3 out of 4 blood samples were used as all of them are from siblings). Amplification of these samples using primers for microsatellite loci was more successful, but sill problematic. Except for the case of the locus Cj128, where the amplification showed to be so poor, that it had to be excluded from further analyses.

4.1. Purebredness

4.1.1. Maternal DNA

The amplification and subsequent gaining of a cytochorme *b* sequence was succesful in all individuals of *C. mindorensis*. For the second species, *C. rhombifer*, 4 individuals were used. The alignment of cyt *b* gene was made for 843 bp and the results are well supported and relationships among crocodile species robustly resolved. These results are present in a strict consensus (L=1137, CI=50, RI=97) of a 10000 trees (L=1062, CI=54, RI=97) (see Appendix). For D-loop the strict consensus (L=877, CI=57, RI=93) of a 10000 trees (L=897, CI=55, RI=92) was made out of a 522 bp long alignment of (Appendix).

Below are shown the detailed sections of the phylogenetic trees obtained by BI with numerical values of the posterior probability (complete trees in Appendix).

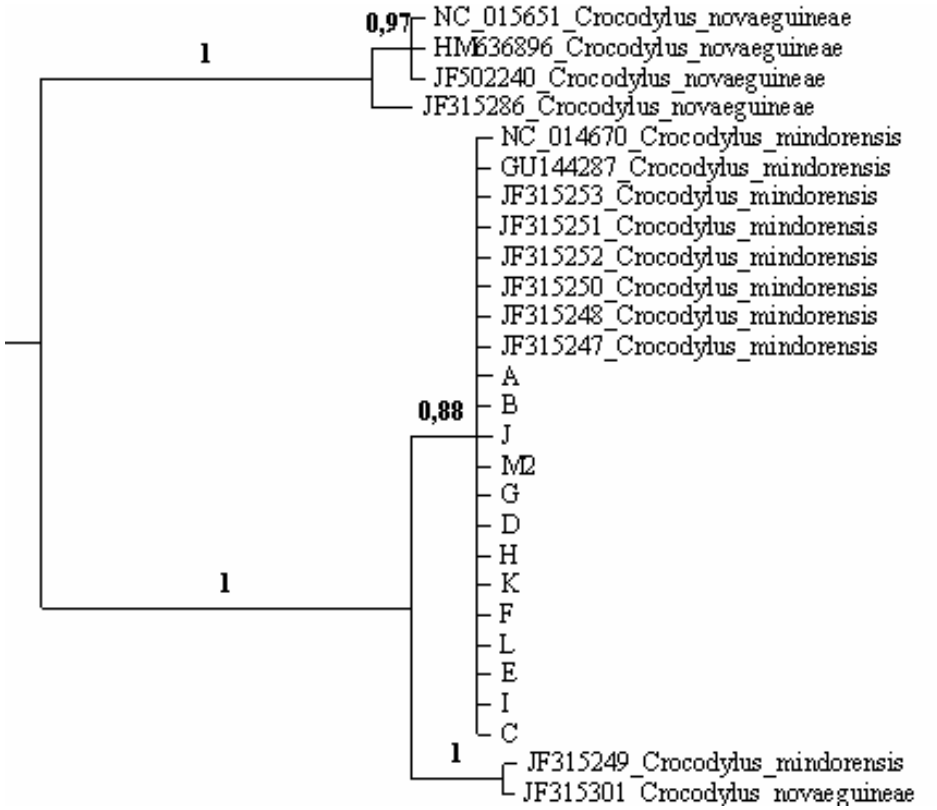

Fig. 4. Detailed section of the phylogenetic tree (BI) for cytochrome *b* showing all *C. mindorensis* from the Crocodile ZOO Protivín.

The Cuban crocodile individuals R, R_1, R_2 and R_3 from the Crocodile ZOO Protivín are placed among *C. rhombifer* cyt *b* sequences (Fig. 5), specifically in a group comprising individuals with the α - haplotype. All Philippine crocodiles from the ZOO Protivín form a monophyletic group with all available mitochondrial cyt *b* sequences of *C. mindorensis*. Additionally, both of these groupings has a robust (100%) support.

Moreover, some GenBank sequences of particular species are associated with sequences of other species, namely JF315301, *C. novaeguineae* has „*minodrensis*“ mtDNA (Fig. 4), several *C. porosus* have „*siamensis*“ mtDNA, JF315276 *C. moreleti* the „*acutus*“ mtDNA sequesnces, and several individuals of *C. acutus* have „*rhombifer*“ mtDNA (Fig. 5). All these cases could be associated with wrongly determined individuals and/or hybrid status of particular individuals and/or ancestral polymorphism resulting in incomplete lineage sorting. Isolate from the *C. novaeguineae* specimen, here coded JF315301 and JF315494 in LDHA tree (Fig. 7) from the work of Oaks (2011) is nested in the *C. mindorensis* clade making it seem paraphyletic.

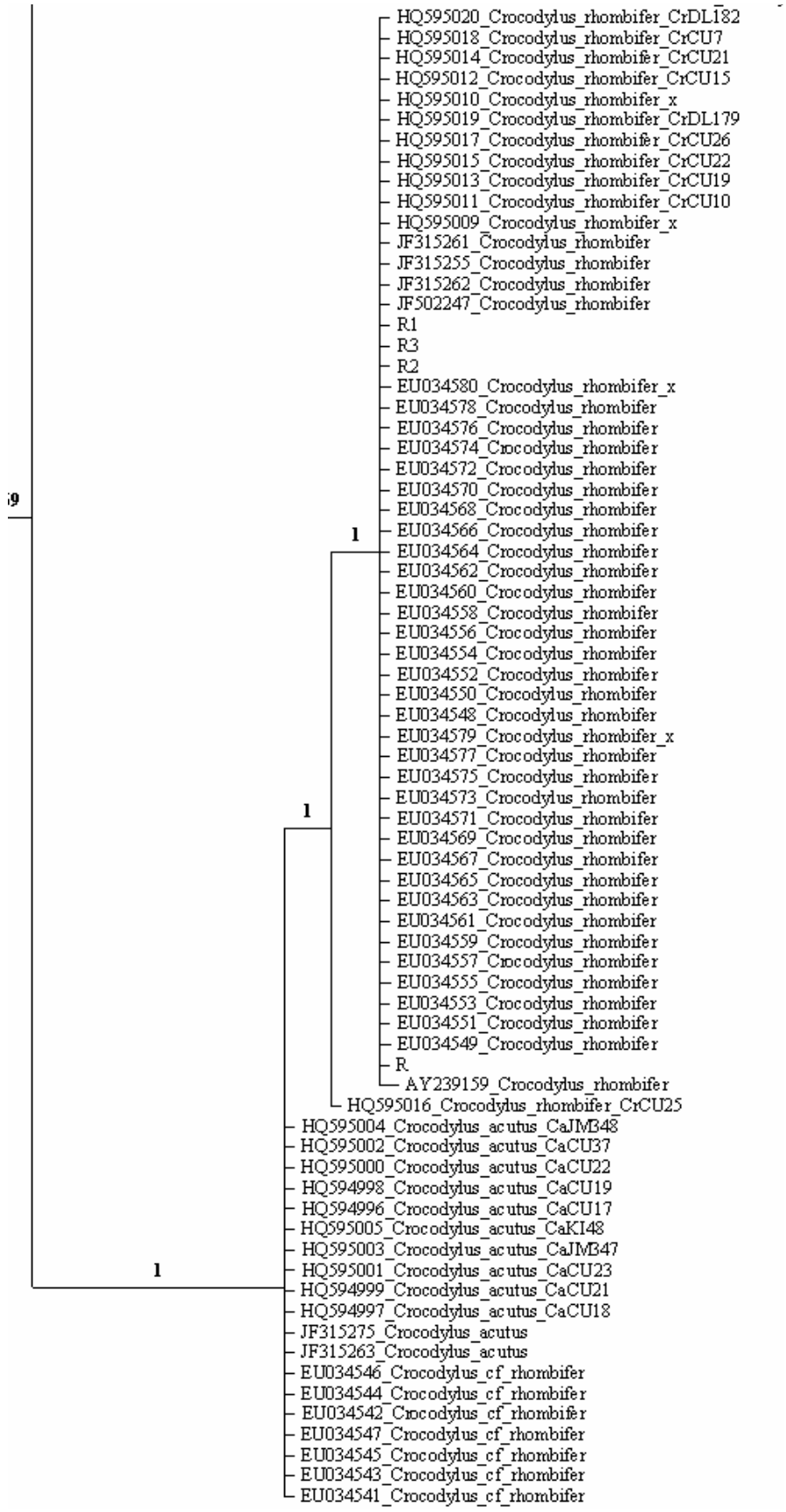

Fig. 5. Detailed section of the tree for cytochrome *b* (BI) showing all *C. rhombifer* from the Crocodile ZOO Protivín. The wole clade (HQ595020 – AY239159) comprises individuals with the α mitochondrial haplotype.

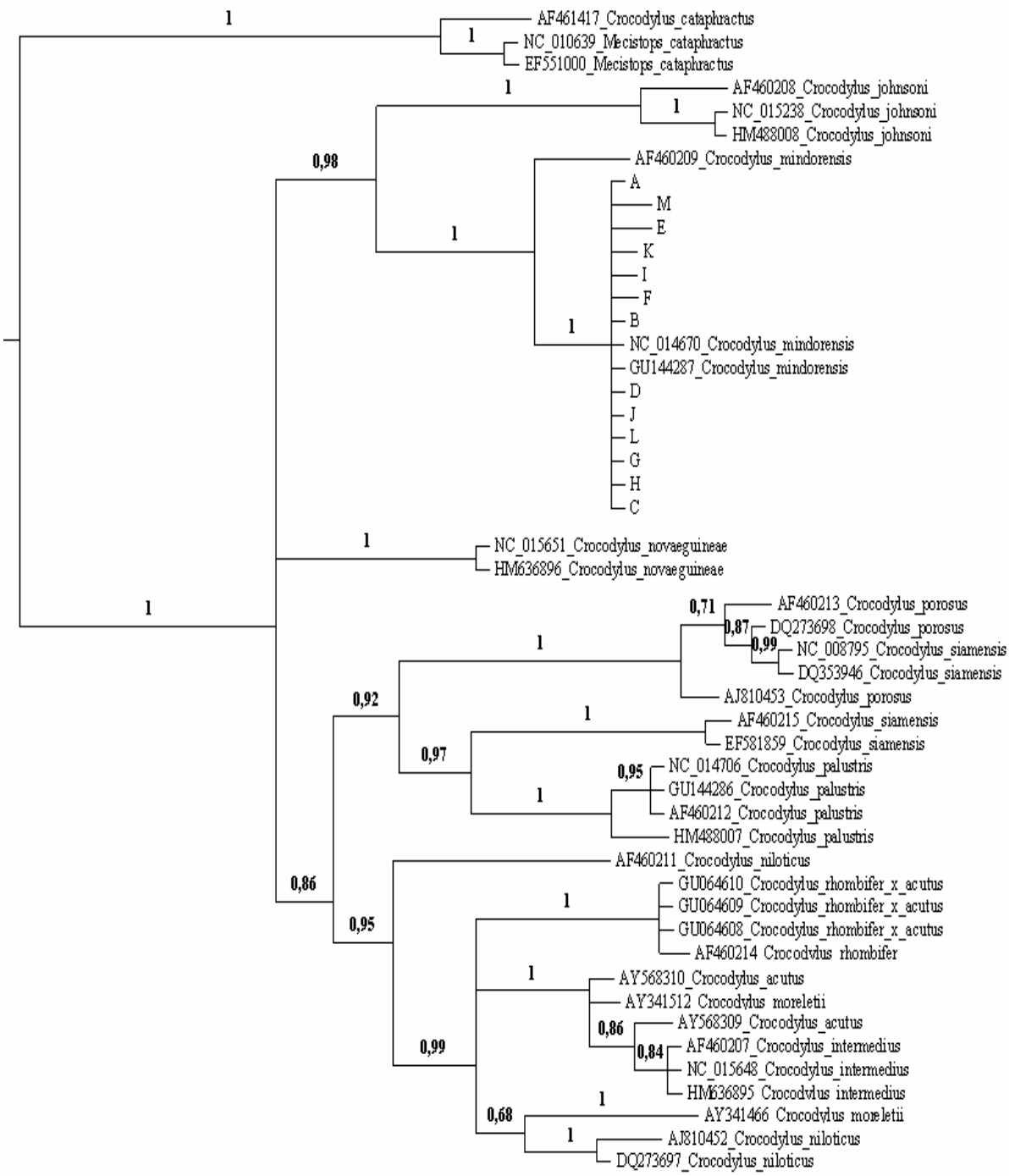

Fig. 6. Section from the phylogenetic tree for a D-loop loci of the control area (BI) showing all *C. mindorensis* from the Crocodile ZOO Protivín.

Both mtDNA markers, cyt *b* and D-loop, have positioned specimen analysed in this thesis into a monophyletic groups of their assigned species. Hence all individuals of *C. mindorensis* and *C. rhombifer* are maternaly purebred. Also both markers succeeded to successfuly distinguish between a closely related species *C. mindorensis* and *C. novaeguineae*. Maximum parsimony analyse provided the same results (Appendix).

4.1.2. Nuclear markers

The LDHA (alignment length 714 bp) results from Maximum parsimony are present in a strict consensus (L=197, CI=82, RI=97) derived from 1089 trees (L=175, CI=92, RI=99). The same applies for the C-*mos* (alignment length 348 bp), where the parameters of a strict consensus are L=48, CI=60 and RI=95 derived from 171 trees (L=33, CI=87, RI=99).

Both of the analysed nucDNA markers grouped all sampled crocodiles with other individuals of their respective species. With LDHA, the Cuban crocodiles form a monophyletic cluster. The monophyly of the *C. mindorensis* clade is disturbed by a single sequence of *C. novaeguineae* (i.e. JF315494) with disputative origin (Discussion). The clade that the Philippine crocodiles form in C-*mos* is paraphyletic, as this gene marker is unable to resolve them from the New Guinea crocodiles. All observed individuals belong to a single group with every available seqence of *C. mindorensis* and do not occur anywhere else. Maximum parsimony analyse provided the same results (Appendix).

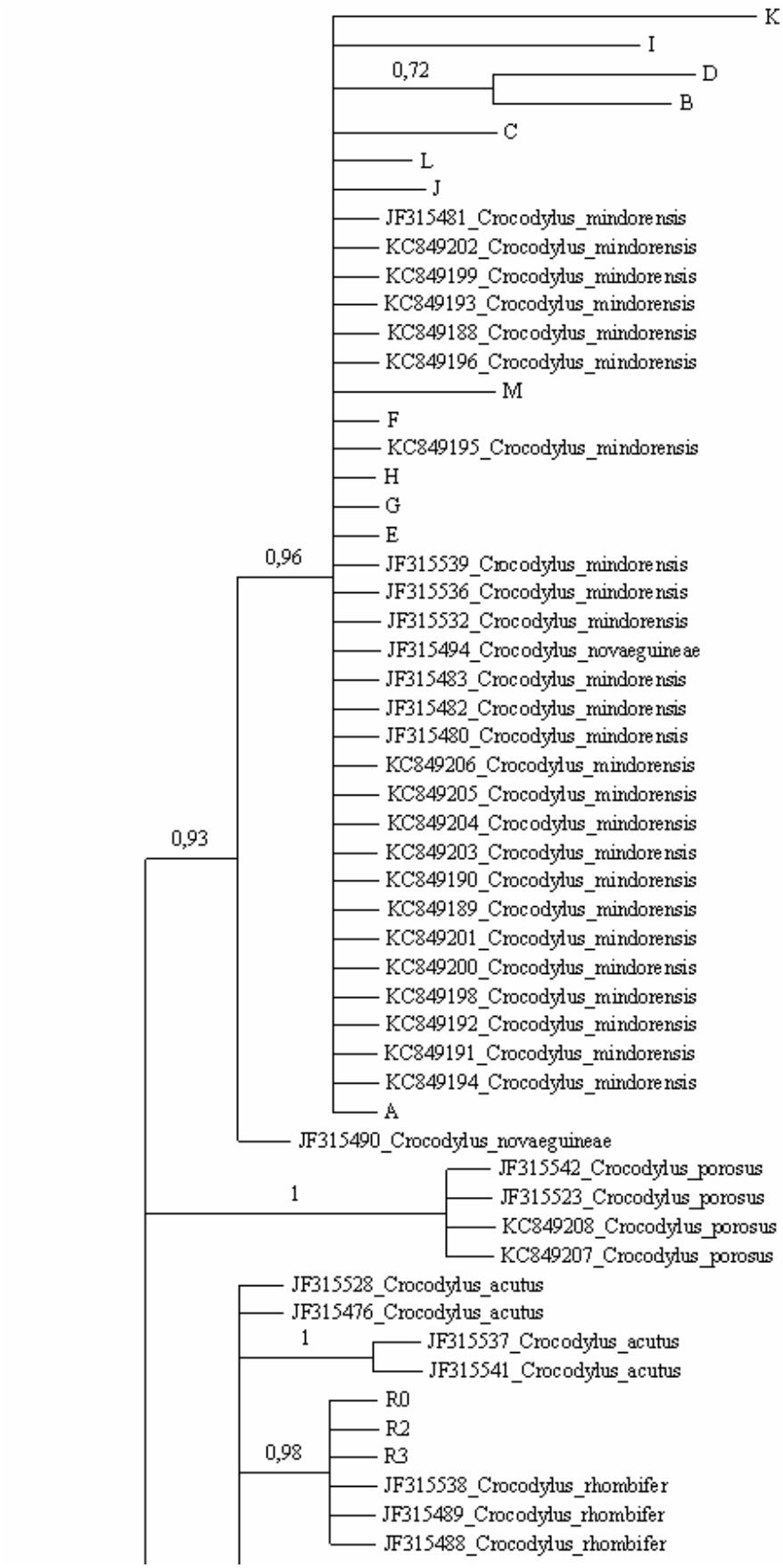

Fig. 7. Detailed section of the phylogenetic tree for the LDHA gene showing individuals from the Crocodile ZOO Protivín.

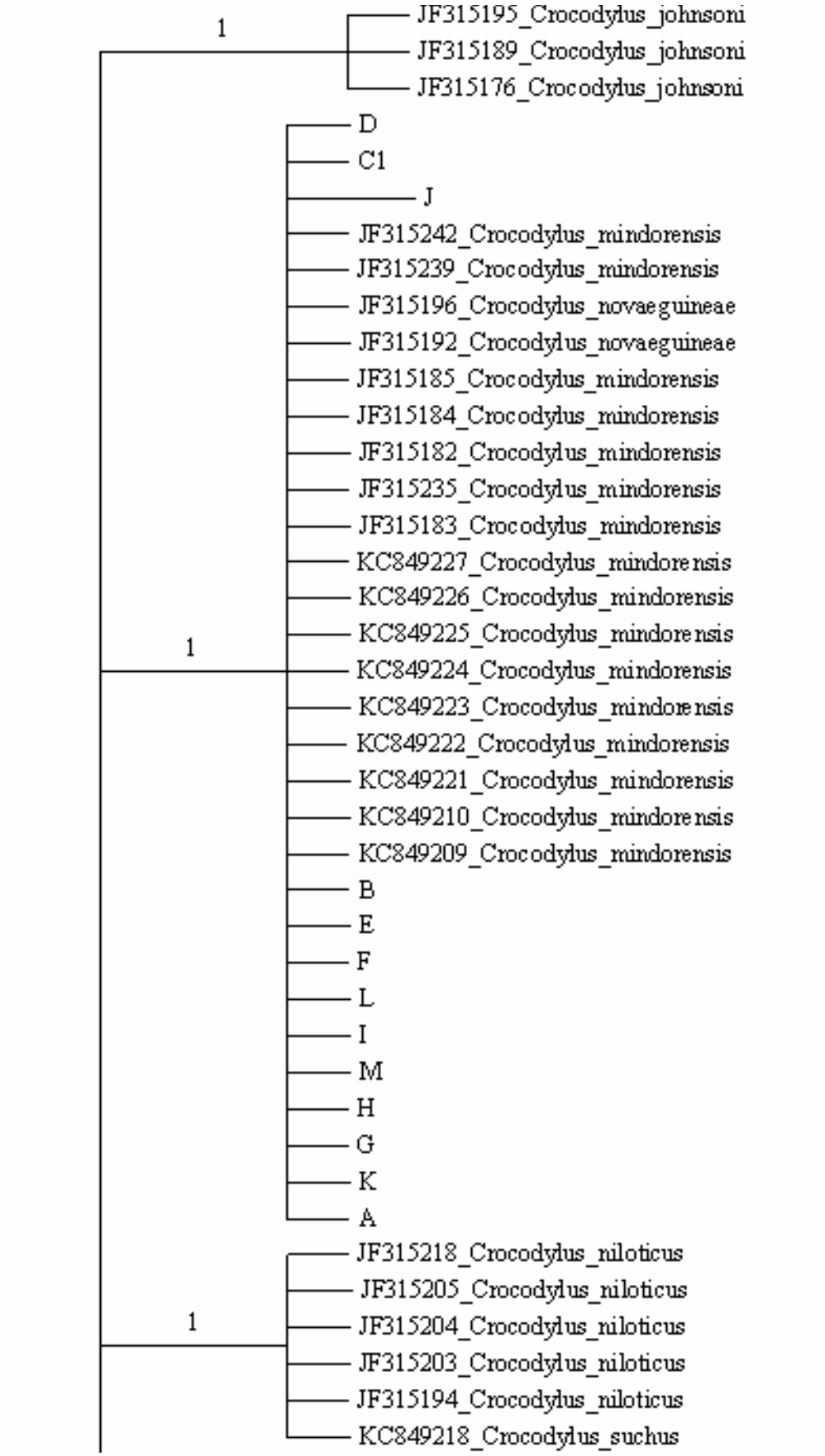

Fig. 8. Detailed section of the phylogenetic tree for the C-*mos* gene showing all *C. mindorensis* individuals from the Crocodile ZOO Protivín.

Among the nuclear genes used above, sequences for potentially hybridizing species (*C. novoguineae* and *C. porosus* for *Crocodylus mindorensis*; *C. acutus* for *Crocodylus rhombifer*) were compared to check the variable sites and help confirming the purebredness of studied individuals. There are 10 variable sites for 5 species of interest (see Tab.2.) in LDHA and only 2 for 3 species of interest (see Tab.3.) in C-*mos*, whereas only one position in LDHA seems to distinguish between closely related *C. mindorensis* and *C. novoguineae* and none in C-*mos* gene (see Discussion). All studied specimen are in agreement with data provided by seqences of concerned species from GenBank database (for table containing studied individuals see Appendix) and that means that no hybrid was found.

species / position	***56***	***100***	***158***	***249***	***297***	***317***	***358***	***369***	***553***	***554***
C.mindorensis	A	A	A	T	A	G	A	A	C	A
C.porosus	A	G	T	C	G	A	A	T	T	G
C.novaeguineae	A	A	T/A	T	A	G	A	A	C	A
C.rhombifer	G	A	T	T	G	G	C	A	C	A
C.acutus	A	A	T	T	G	G	C	A	C	A

Tab.2. Variable sites for LDHA gene in our two studied species and their most probable hybridizing species. The conflict in third site in *C. novaeguineae* is caused by already mentioned isolate JF315494 (Discussion)

species / position	***109***	***118***
C.mindorensis	A	A
C.porosus	G	G
C.novaeguineae	A	A

Tab.3. Variable sites for C-*mos* gene in our two studied species and their most probable hybridizing species.

4.1.3. Microsatellite analyses

Philippine crocodiles:

From a number of microsatellite primers designed for various crocodile species by Fitzsimmons et al. (2000), 7 potentionally diagnostic for *C. mindorensis* were selected. Because these were not originally developed specifically for the Philippine crocodiles, the successful amplification of some of them is limited (Tab. 4). Only one locus has worked with the individuals „G“ and „H“.

All individuals either fall into the alele base range proposed by Fitzsimmons et al. (2000), are very close to it (closer than to another species) or if not, their values are constant in more specimen. Microsatellite variability derives accordingly to the number of analysed individuals of species/populations, so the allele values occurring outside ranges detected by Fitsimmons et al. (2000) may be a natural, not yet detected variability of the purebred individuals.

primer	**C391**	**CU5-123**	**CUD78**	**Cj18**	**CUJ 131**	**CR52**	**CUI99.2**
expected range	*127-159*	*198-228*	*155-209*	*200-214*	*183-187/262-264*	*109-151*	*164/184-216*
A	142/146	190/200	201/201	190/200	223/231	190/200	166/166
B	142/146	200/200	201/201	190/200	223/231	190/200	166/166
C	142/146	188/198	199/199	190/200	223/233	190/200	166/166
D	142/146	200/200	199/199	190/200	201/201	190/200	166/166
E	142/146	200/200	199/199	190/200	201/201	190/200	166/166
F	132/142	200/200	199/199	200/200	223/231	200/200	164/168
G	/	/	/	/	/	/	166/166
H	/	/	/	/	/	/	166/166
I	140/146	190/200	191/201	190/200	223/231	224/224	154/168
J	142/146	190/200	191/201	190/200	223/231	224/232	154/168
K	140/140	190/200	191/201	190/200	223/231	/	154/168
L	142/142	190/200	201/201	190/200	223/231	224/232	154/168
M	142/146	200/200	199/199	200/208	/	/	/

Tab. 4. Summary of used primers, their expected allele range (according to Fitzsimmons et al., 2000) and values obtained for each individual. Where there is no value shown, the amplification was unsuccessful

The obtained values of allele range make possible to consider specimen A-F and I - L to be purebred individuals of *C. mindorensis* based on available loci. Although the individual M amplified only in some of the loci, in all of these fits into expected ranges and is the same, or at least very close to the other probably purebred crocodiles. As for the specimen G and H the situation differs.

Cuban crocodiles:

14 loci them were chosen for this work but one was excluded after a very poor amplification (Tab. 5). Individuals R_4, R_5 and R_6 have not amplify for locus CU5-123, otherwise all reactions provided some value for an allele range.

primer	**Cj 16**	**Cj 18**	**Cj 35**	**Cj 101**	**Cj 104**	**Cj 109**	**Cj 119**
expected range	*140-166*	*200-327*	*167-189*	*364*	*209-217*	*368-419*	*176-217*
R_0	147/161	200/210	148/160	360	209/217	348	183
R_1	147/161	210/210	148/148	360	209/217	348	183
R_2	147/161	210/210	148/148	360	209/217	348	183
R_3	147/161	210/210	148/148	360	209/217	348	183
R_4	145/145	210/210	148/160	360	217/217	348	183
R_5	147/147	200/210	148/148	360	213/213	348	183
R_6	147/161	200/212	148/148	360	215/215	348	183

primer	**Cj 127**	**Cj 131**	**Cp 10**	**Cr 391**	**CU5-123**	**CUJ 131**
expected range	*331-425*	*178-264*	*192*	*126-185*	*198-233*	*173-264*
R_0	318	245	166/174	126/134	207/215	210
R_1	320	245	166/174	126/134	207/215	210
R_2	320	245	166/174	126/126	203/215	210
R_3	320	245	166/174	126/134	203/207	210
R_4	318	245	166/174	126/126	/	216
R_5	320	245	166/166	126/134	/	214
R_6	318	245	164/166	126/132	/	214

Tab. 5. Summary of used primers, their expected allele range (accordinng to Fitzsimmons et al., 2000; 2002; Weaver et al., 2008; Milián-García et al., 2011) and values obtained for each individual. Where there is no value shown, the amplification was unsuccessful.

The obtained allele values mostly lies in their expected ranges. In situations, where this is not true, the observed figures are at least very close to the ones taken from other studies (Fitsimmons et al., 2000; Milián-García et al., 2011; Weaver et al., 2008) and differ from other crocodile species. As only a small portion of population was sampled untill today, there is still a high possibility that these markers have much broader variability and these results seem to be the case.

4.2. Kinship

4.2.1. *Crocodylus mindorensis*

The outcome of the Fragment analysis was proceed with the program ML Relate (see Materials and methods). Individuals G and H do not occur in this analysis for they did not amplify for most of the loci.

	A	**B**	**C**	**D**	**E**	**F**	**I**	**J**	**K**	**L**	**M**
A	1										
B	0,8	1									
C	0,39	0,39	1								
D	0,2	0,36	0,41	1							
E	0,39	0,57	0,6	0,87	1						
F	0	0	0	0	0	1					
I	0,14	0	0	0	0	0	1				
J	0,36	0,14	0	0	0	0	0,78	1			
K	0,24	0	0	0	0	0	0,87	0,78	1		
L	0,4	0,21	0	0	0	0	0,49	0,79	0,54	1	
M	0	0,19	0,33	0,4	0,63	0,41	0	0	0	0	1

Tab. 6. Matrix of maximum likelihood relatedness. Numbers here represents the coefficient of relatedness (r).

	A	**B**	**C**	**D**	**E**	**F**	**I**	**J**	**K**	**L**	**M**
A	-										
B	FS	-									
C	U	U	-								
D	U	U	U	-							
E	U	FS	FS	FS	-						
F	U	U	U	U	U	-					
I	U	U	U	U	U	U	-				
J	U	U	U	U	U	U	FS	-			
K	U	U	U	U	U	U	FS	FS	-		
L	U	U	U	U	U	U	FS	FS	FS	-	
M	U	U	U	PO	FS	PO	U	U	U	U	-

Tab. 7. Matrix of relationships suggesting kinship between studied individuals.

Based on this program´s suggestion, the combination of A with B, C, E or J; B with C, D or E; C with D or E; D with E or M; E with M; F with M; I with J, K or L; and K with L;

should be rejected or left as the last possibility (as in some combinations the values are considerably high but not alarming). All other combinations would be beneficial (i.e. A with D, F, I, K and M; B with all individuals except for A, B and C; C with all individuals except for D; D with A, F, I, K, L; E with F, I, J, K, L; F with all except for M; I with all except for J, K, L; J with B, C, D, E, F, M; K with all except for I, J, K; and L with B, C, D, E, F and M) for retaining of genetic variabiality in *ex situ* conservation of this species.

4.2.2. *Crocodylus rhombifer*

	R0	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**
R0	1						
R1	0,51	1					
R2	0,19	0,63	1				
R3	0,34	0,82	0,76	1			
R4	0,17	0	0	0	1		
R5	0	0	0	0	0	1	
R6	0,01	0	0	0	0	0,07	1

Tab. 8. Matrix of maximum likelihood relatedness with calculated values of the coefficient of relatedness.

	R0	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**
R0	-						
R1	FS	-					
R2	U	FS	-				
R3	U	FS	FS	-			
R4	U	U	U	U	-		
R5	U	U	U	U	U	-	
R6	U	U	U	U	U	U	-

Tab. 9. Matrix of relationships suggesting kinship between studied individuals.

Relationships resulting from this analysis prefer breeding of individuals R_4, R_5 and R_6 in any combination, whereas R_1, R_2 and R_3 share a considerable similarity in their genetic profile. Eventhough it seems that R_0 is unrelated to R_2 and R_3, these two came out as siblings to each other as well as to R_1, which, on the other hand, stand here as a sibling to R_0.

5. DISCUSSION

5.1. Using of a cross-species molecular markers

One of the possible problems that could be anticipated in the pursuance of the laboratory part of this work was the selection of suitable and functional primers. This is not as much the case in *C. rhombifer*, as this was a target species in several published studies, mostly dealing with detecting hybrids with the American crocodile *C. acutus* in areas of their sympatric occurrence (Weaver et al., 2008; Milián-García et al., 2011). On the other hand, there have not been many surveys conducted for the Philippine crocodile. Therefore the necessity of trying a cross-species markers has emerged.

There are several works that aimed to prove the multispecies application of primers designed for only a few or even a single one target crocodile species, mainly Fitzsimmons et al. (2000), Weaver et al. (2008) and Miles et al. (2009). The authors of these studies have shown that the majority of primers for mitochondrial and nuclear genes or microsatellite loci, originally proposed for specific species, can be successfully used in most of them. This agrees with the results of this thesis, as the primers chosen here worked quite well for all of the amplified samples (with only a few limitations where a slight alternation in PRC condition was needed).

Comparing the obtained data with the ones previously published by other authors and thus suggesting valid conclusions proved to be much bigger difficulty, especially with the microsatellite loci. Sequences of mitochondrial and nuclear genes are placed in the GenBank in an amount and variety that allows the phylogenetic analyses to build a well supported cladograms. Yet the situation for microsatellites is a lot worse than it could be expected from the wide and frequent usage of the these markers. The fundamental study of Fitzsimmons et al. (2000) tested on only two individuals of *C. mindorensis*, and Miles et al. (2009) tested quite a numerous amount of primers but on just a single one Philippine crocodile.

Eventhough the Cuban crocodiles do appear much more often in the genetic studies, the quantity of individuals used there is still desperately small. In works where *C. rhombifer* was a targeted species (i.e. Weaver et al., 2008; Milián-García et al., 2011), the number

of samples were 30 and 17, respectively. Miles et al. (2009), who have tested microsatellite loci designed for *C. porosus* in 18 non-targeted species, examined only 3 individuals of the Cuban crocodile, whereas Fitzimmons et al. (2000) have tried the usage of cross-species primers on 10 crocodiles belonging to *C. rhombifer*.

Also the certainty of the purebredness of individuals from various works, whether sampled from the wild or captive stock, occurring in the GenBank and other databases might not always be absolute as more and more hybrids are found in population considered purebred using more subtle surveys.

Microsatellites:

More than a half of the chosen loci for each species, tested in this thesis, fits into the allele range expected based on previously mentioned studies; yet some of them does not (3/7 loci for *C. mindorensis* and 5/13 in *C. rhombifer*). The fact that all (or most of the) individuals share the same or significantly close allele sizes when differing from the expected value, indicates that it is unlikely to be by mistake, but rather as a consequence of varying monomorphy/polymorphy and allele range. This inconsistence could mean either that the sampled individuals have a hybrid origin or that to date known values of allele sizes are not definite and would broaden with increasing number of sampled specimen. Given the fact that values obtained in this experimental study are identical or very similar in all individuals and at the same time are closer to the purebred targeted species than to any other, the second hypothesis seems more likely. It is especially the case in *C. mindorensis*, where the sampled group is considerably larger than the number of individuals that gave the expected allele range in the first place.

Therefore the suggestion, that not only do my results support the species purity with respect to the nuclear DNA, but have also detected a wider variability of alleles in regarded microsatellite loci. This is the true for 11 out of 13 individuals of examined Philippine crocodiles. The remaining two (samples G and H) did amplify for only one locus and so there is a hesitation with proclaiming them as being purebred. Anyway, it would correspond with a supposition about their hybrid origin, based on their morphotype (pattern of the ventral scales). The reason for only a single locus amplification could be a failed PCR, but as the DNA extraction and PCR were repeated (several times) and the same isolates gave

results for the other markers, it does not seem likely. Still, the fact of not amplifying at all is questionable, as with hybrid origin at least a different alleles should emerge.

Although the specimen of *C. rhombifer*, used in this thesis, is smaller compared to the ones from previous studies (Fitzsimmons et al., 2000; Weaver et al., 2008; Milián-García et al., 2011), the possibility of revealing a broader variability in allele size range is still significantly high. Regarding the same arguments as in the case of the Philippine crocodile and a higher number of diagnostic loci, all of the Cuban crocodile specimen could be considered as purebred.

Mitochondial DNA markers:

The analysis of the mitochondrial DNA, specifically the cytochrome *b* gene using primers proposed by Weaver et al. (2008), gave out results congruent with the expected hypothesis. All individuals belonging to *C. rhombifer* are supposed to have been maternaly pure. The cladogram resulting from comparison of the sequence gained from 4 here tested specimen and others from GenBank database, agrees with this initial assumption. Furthermore, the Cuban crocodiles from the Crocodile ZOO Protivín belong with a group joined by an α-haplotype indicating no signs of even historical introgression or hybridization with the American crocodile (Milián-García et al., 2011).

Samples from *C. mindorensis* formed a monophyletic group of a pure blooded Philippine crocodiles. The individuals examined under labels G and H came out purebred from both markers for the maternal side and also two nuclear genes. Yet they do exhibit the morphotype of a mixed origin with *C. mindorensis* being one of the parent species. The unsuccessful amplification of the nuclear microsatellite loci may follow the supposed hypothesis that they might possess genes from another species (probably *C. novaeguineae* from the scale pattern). Still, none of the present results can actually confirm this hypothesis. The reason for their apparent purebredness for all markers except for the microsatellite loci could perhaps be the incompetence of some of them to distinguish between so closely related species as are *C. mindorensis* and *C. novaeguineae*. In further work, we would like to test a few new loci that have been examined for both of the species and so could possibly resolve this issue.

Nuclear DNA markers:

C-*mos* marker do not differ between *C. mindorensis* and *C. novaeguineae* and so resulted in a paraphyletic group of these two species, but gathering all Philippine crocodiles. The monophyly of *C. mindorensis* cluster based on LDHA was disrupted by a single *C. novaeguineae* sequence coming from the same isolate as in a cytochrome *b*, where it is nested within the otherwise monophyletic group or suspiciously near, respectively. This individual (isolate number LSUMZ H-6995) appears in the work of Oaks (2011), where he suggests that this unusuall positioning could be either a result of the existence of *C. mindorensis* population in New Guinea (hence the determination as a New Guinea crocodile) or that this could be a by-product caused by a captive keeping of this individual associated with origin that might not be absolutely clear.

The assessment of genetic purity of the captive bred crocodiles is highly necessary, as it would allow to exclude hybrids from breeding and subsequent introducing animals to reinforce wild populations. Out of the two species concerned in this study this probably is more urgent for *C. rhombifer*, as it tend to hybridize much more often than previously thought. This hybridization occurs in natural population as well as in captive ones. Weaver et al. (2008) found out, that five out of seven captive stocks of the Cuban crocodile in US surveyed had some level of genetic admixture. However, a recent study of Tabora at al. (2012) discovered a concerning portion of *C. mindorensis*/*C. porosus* hybrids in a group of presumably purebred individuals intended to be reintroduced, so the urge probably is the same in both species.

5.2. Effectiveness of used tools for conservation breeding management

The results from the Fragment analyses of microsatellite loci and subsequent outcome from ML Relate programme could provide an important guideline for mating of a convenient individuals while maintaining maximal genetic diversity possible for *ex-situ* conservation breeding (Frankham et al., 2003). It is especially so, when the origin and potential relatedness of individuals is unknown. Sadly, that very often is the case of various breeding farms. The best way to determine justification of the use of this tool is, quite paradoxically, to cross-check the proposed relations with some real ones from groups of known origin and relatedness.

The matrix of relationships propounded for *C. mindorensis* (Tab. 7) assumed a sibling relationship (i.e. resemblance in the genetic profile accord with individuals sharing both parents) between individuals coded I, J, K and L that is possibly consistent with reality (the breeding facility claims that these crocodiles are from one clutch). This matrix suggests some level of kinship in others as well, but it can not be confronted with the real origin as it is not known. The majority of the whole group came from the same farm without believable records and so there is a possibility they could be related. It seems to be the case for the specimen group M, D, E and F or specimen group B, C, D and E. On the other hand, the cause for quite high values of the coefficient of relatedness may be the fact that the population kept in this institution, initially only had a small genetic variability, which might have resulted in a similarity without the need for an actual relationship.

The matrix for *C. rhombifer* is also partially consistent with the real kinship. According to known records, R_0, R_1, R_2 and R_3 are full siblings and R_4 with R_5 are their parents. Whereas all the siblings came out related (directly or through another one of the sibling group), their parents do not show any relatedness towards their offspring (except for R_4 to R_0, but the value of r is not much significant. Because all previously mentioned individuals are of a known relationship, the important point of this analyses was to assign the genetic profile of the specimen R_6, that has been imported from a wild population and therefore should not be related to any of the others. That is in congruence with here presented results, but they still has to be treated with caution because of the questionable DNA quality of the sample. The missing relationship between parents and their progeny is most likely caused by inefficient DNA sampling via mouth swabs.

Eventhough this method is less invasive and easier to execute, it is also prone to contamination (either by a poluted water or by contact with other individuals living in the same water pool) and an actual wash out of the DNA during the sole process of its extraction. Based on that we do suggest gaining samples this way as unsuitable for such analyses. But the blood samples for R_4, R_5 and R_6 should be available soon and so allow better and more precise results.

It is important to realise that it is possible that full or half siblings proposed by the matrix are not necessarily full or half siblings in reality. The reason for this being that this matrix shows relative relationships based on detected variabilites using very sensitive

genetic markers. The reduced genetic variability in endangered species, usually occurring in size limited populations, could shift the „variability spectra“ into a stronger homogenity and „more intensive kin relationships“. This simply stands for the fact that the specimen can have a close and similiar genetic profile without being actually relatives. It is perhaps better to look at the precise values of the coefficient of relatedness as it shows more subtle proximity of genotypes than do the 4 types of relationships. And based on this thesis it is also important to make sure that the DNA sample and subsequent data are absolutely reliable and „artefact-free“, to avoid false suggestions.

Nevertheless, the „matrix of relationships“ and the „matrix of maximum likelihood relatedness“ are very important tools for the breeding management. However, an optimal approach to deal with the *ex-situ* breeding should definitely be a combination of a thorough genetic identification using multiple markers and genes with precise records of the kiship and origin of every single captive crocodile.

6. CONCLUSIONS

I have tested and confirmed the species purity of 13 specimen of *C. mindorensis* on the base of maternally inherited mitochondrial gene cytochrome *b*, mtDNA loci D-loop and two nuclear genes (LDHA and C-*mos*) as well as the purity of 11 out of mentioned 13 individuals on the basis of several microsatellite loci. The two remaining individuals were suspected for the hybrid origin, which this study could neither disprove nor confirm. Then I have verified the purity of 4 of the Cuban crocodile with cyt *b* gene and nuclear purity of 4 tested individuals with LDHA and all 7 individuals with microsatellites.

Based on the obtained genetic characters I proceeded a likely kinship of the two groups of crocodiles and subsequently suggested an optimal combination for breeding management within the *ex-situ* conservation.

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9. APPENDIX

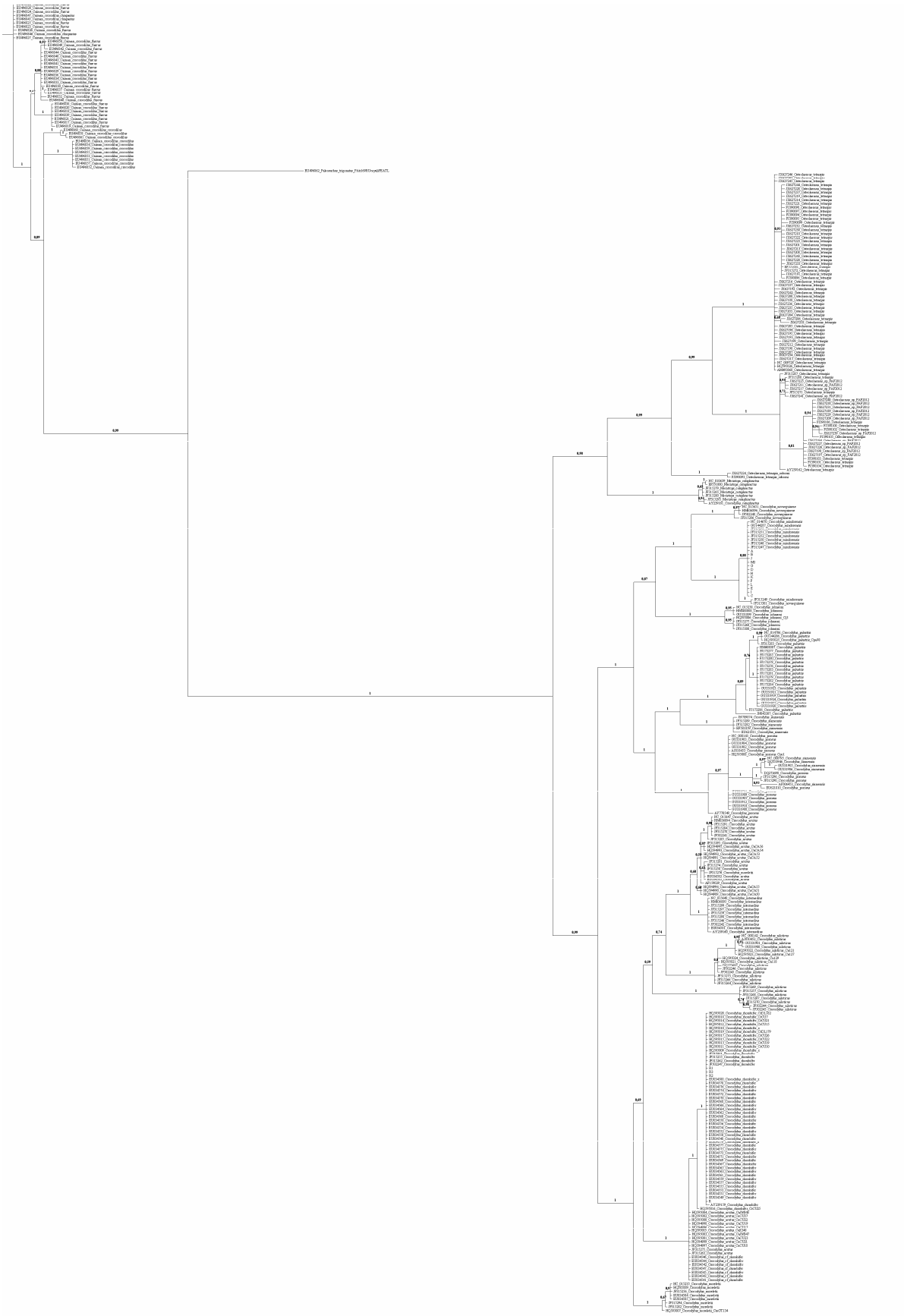

Fig. 1A. A complete phylogenetical tree for cytochrome *b* (BI).

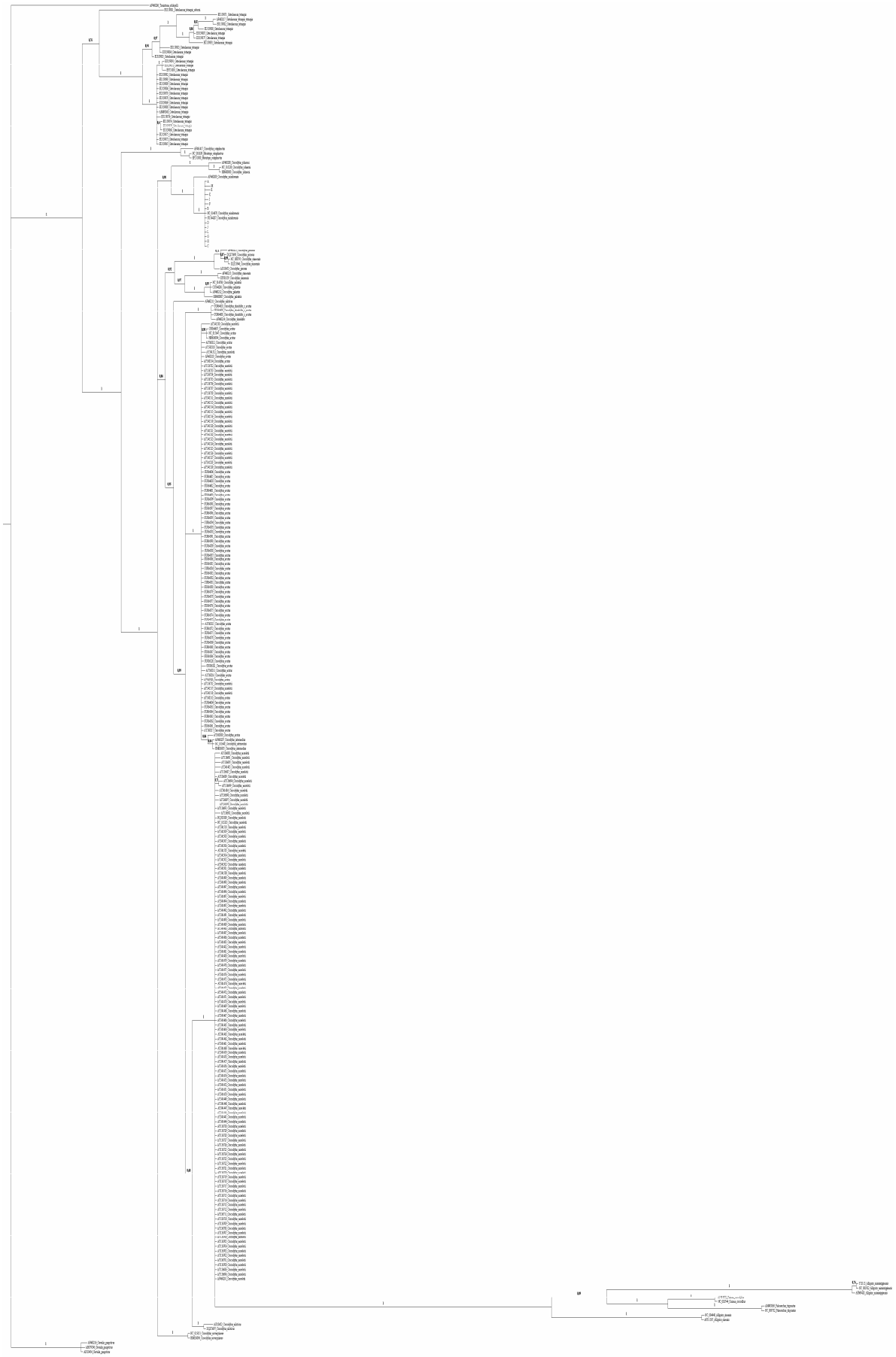

Fig. 2A. A complete phylogenetic tree for D-loop (BI).

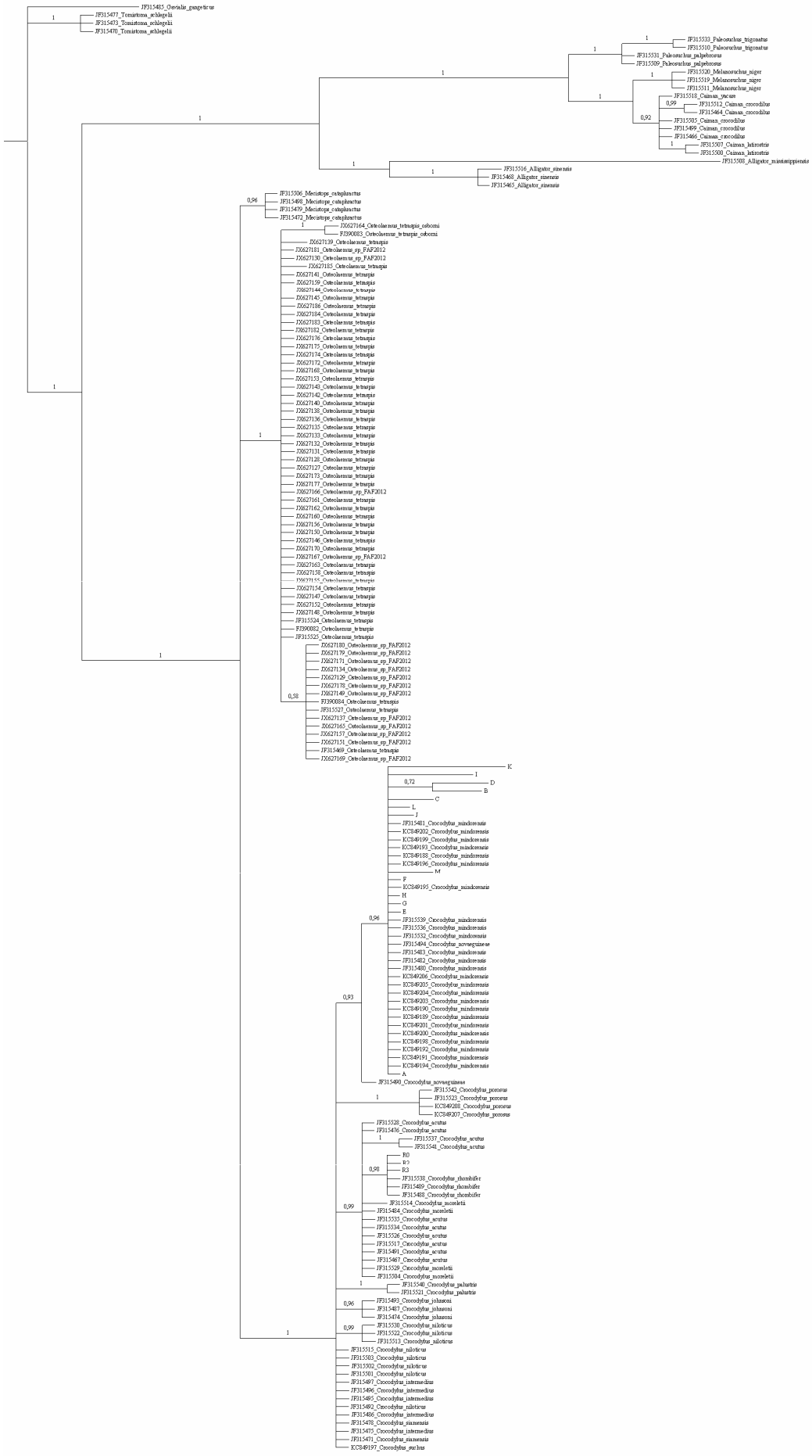

Fig. 3A. A complete phylogenetic tree for LDHA (BI).

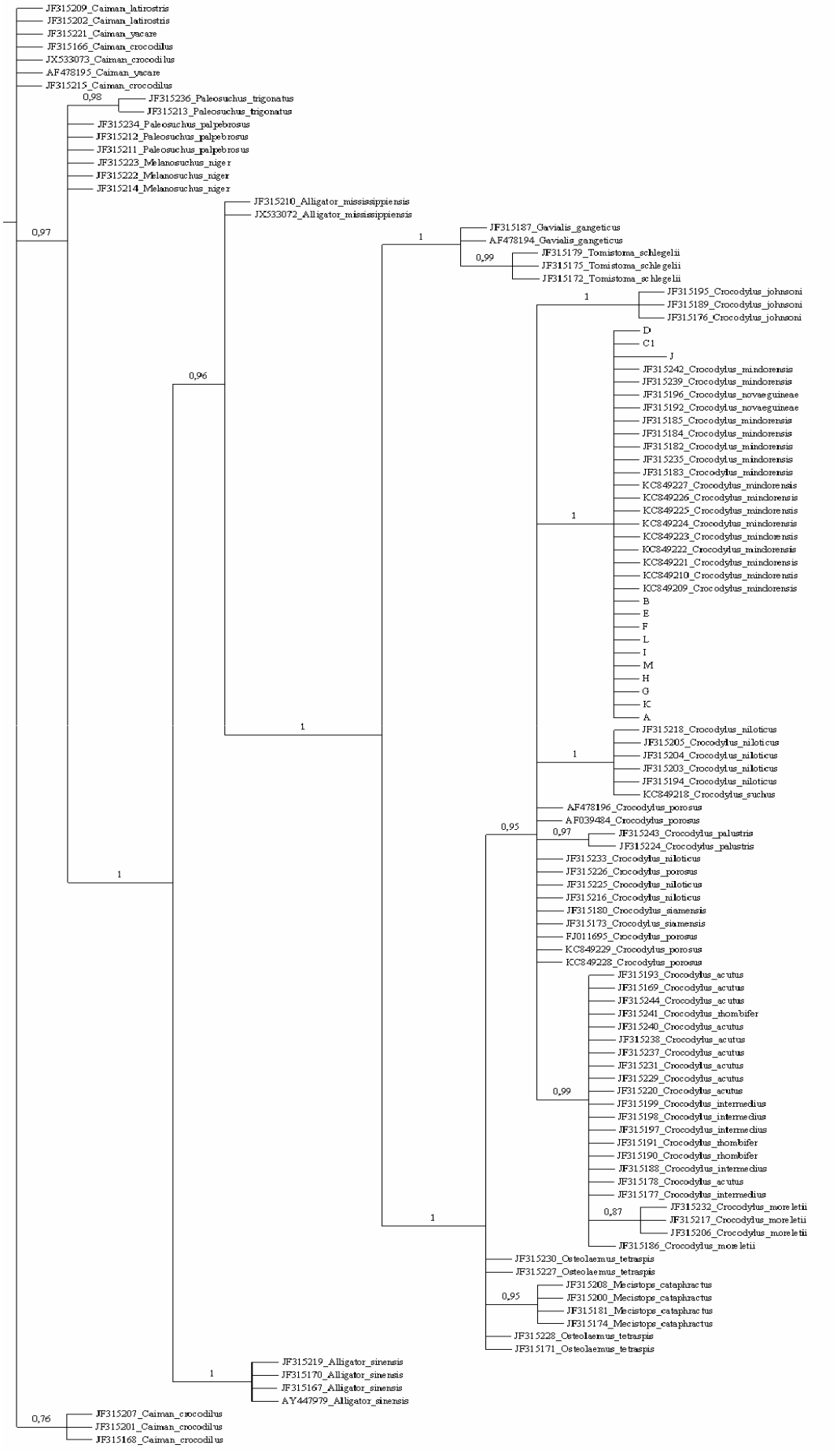

Fig. 4A. A complete phylogenetic tree for C-*mos* (BI).

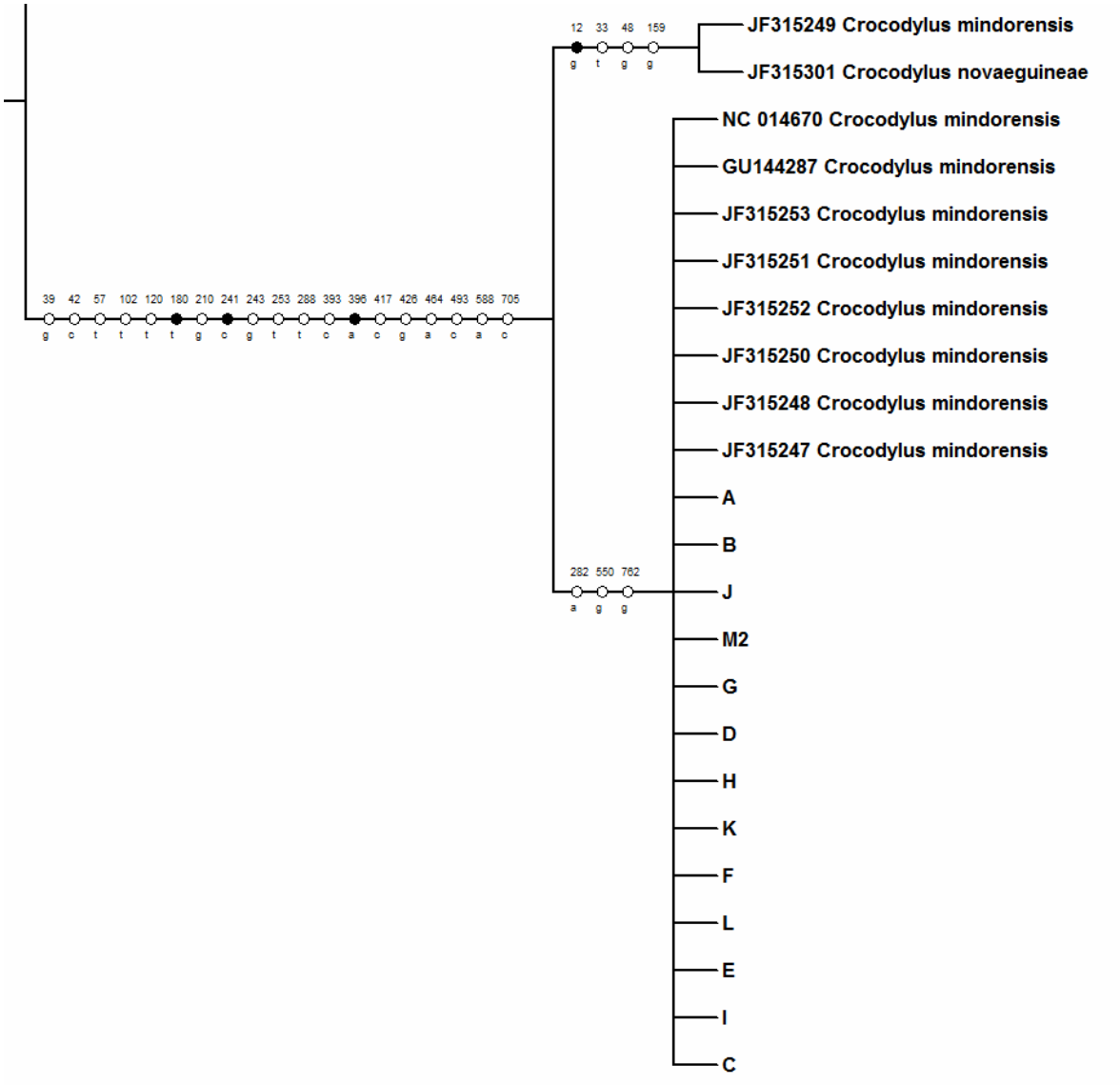

Fig. 5A. A section from a strict consensus (MP) for cytochrome *b* with variable sites. All *C. mindorensis* from the Crocodile ZOO Protivín can be seen.

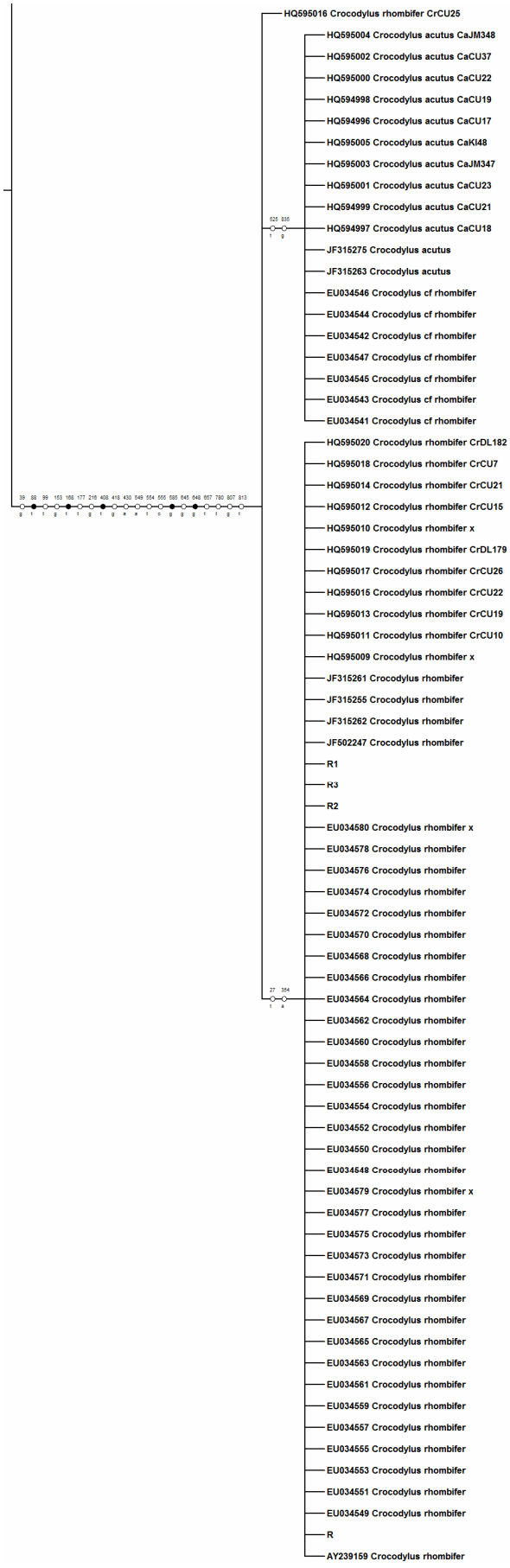

Fig. 6A. A section from a strict consensus (MP) for cytochrome *b* with variable sites. The bottom clade comprises *C. rhombifer* individual with mtDNA haplotype α, the upper with haplotype β.

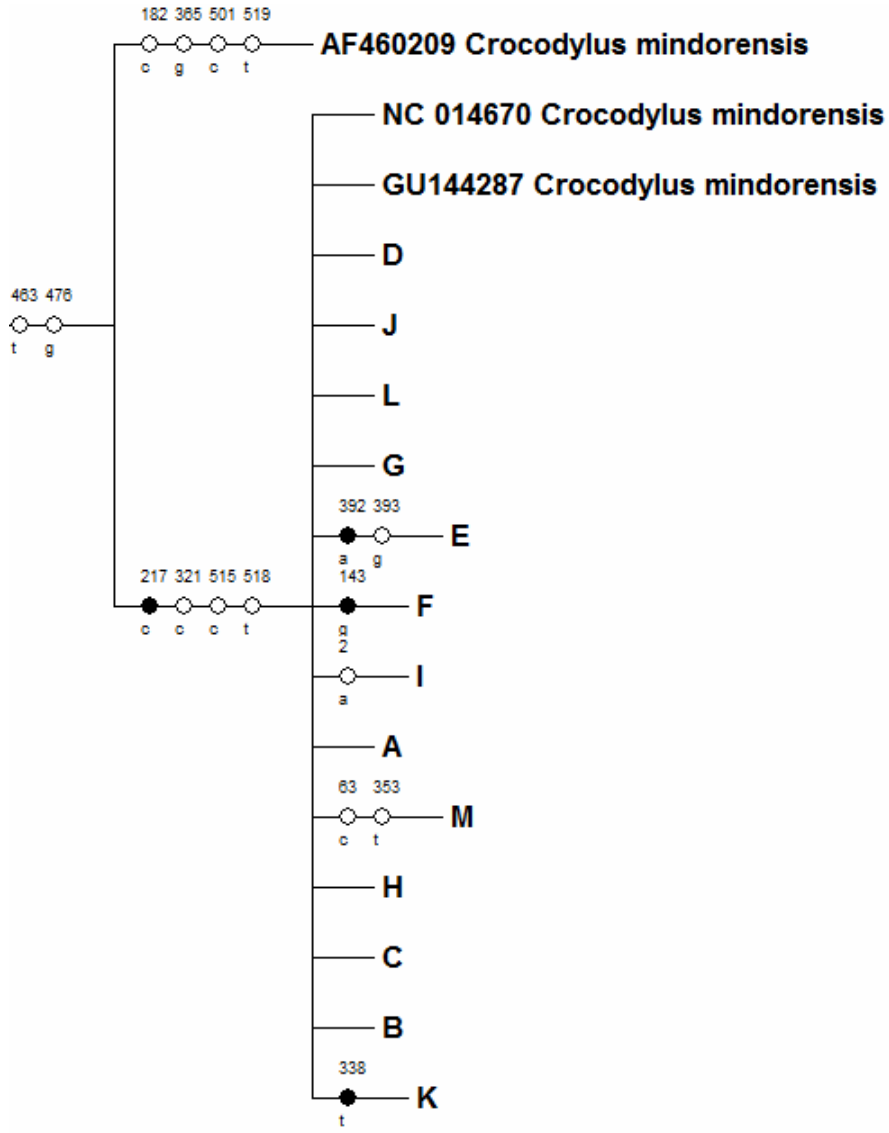

Fig. 7A. A section from a strict consensus (MP) for D-loop with variable sites. All *C. mindorensis* from the Crocodile ZOO Protivín can be seen.

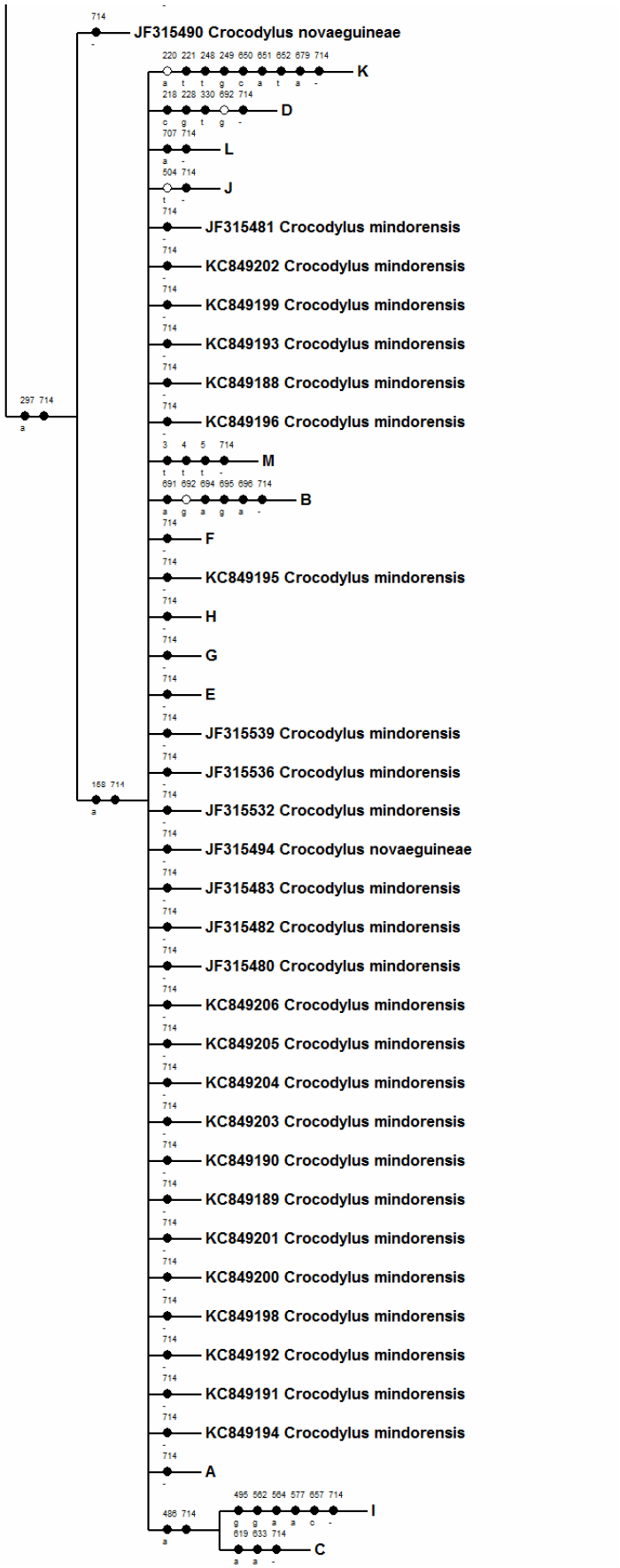

Fig. 8A. A section from a strict consensus (MP) for LDHA with variable sites. All *C. mindorensis* from the Crocodile ZOO Protivín can be seen.

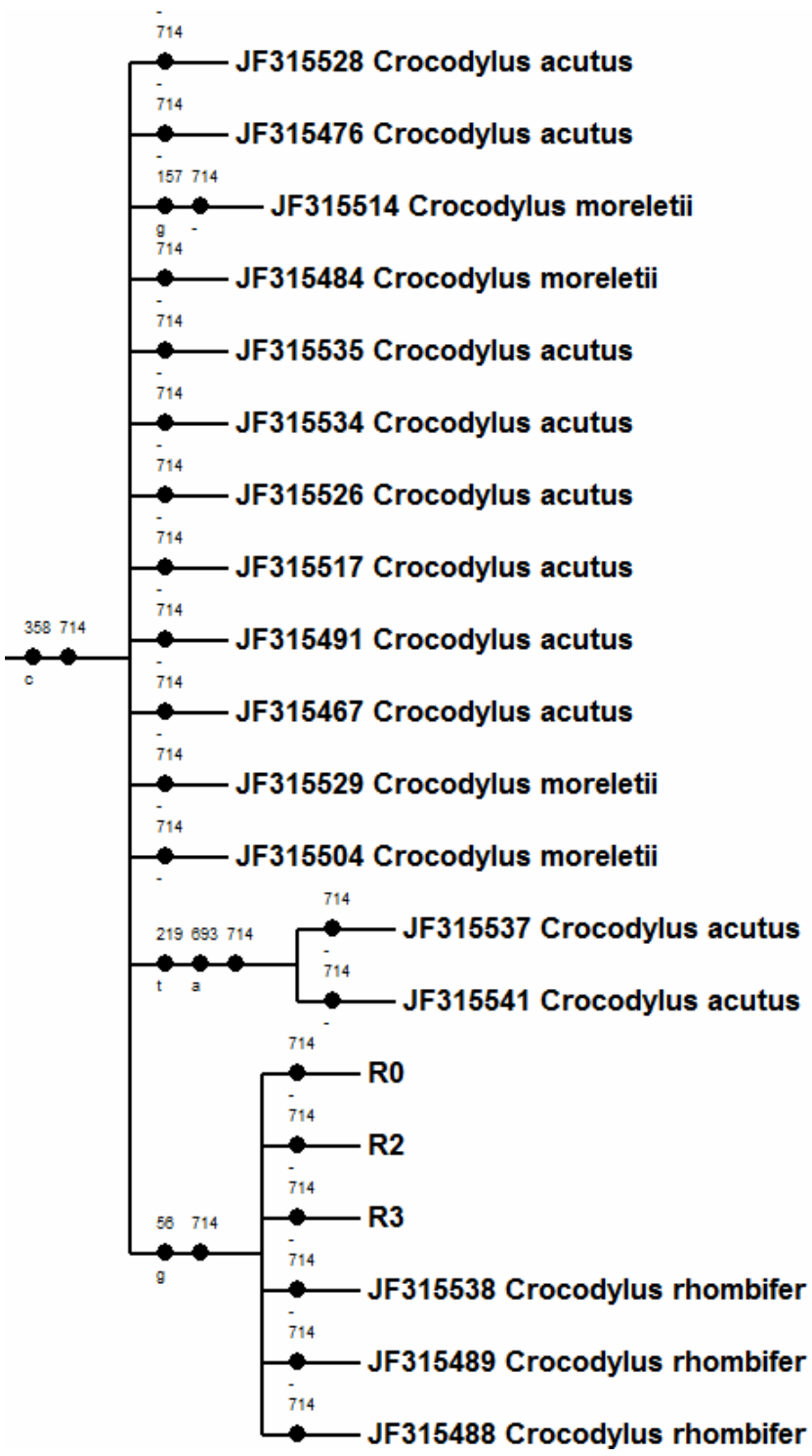

Fig. 9A. A section from a strict consensus (MP) for LDHA with variable sites. All *C.rhombifer* from the Crocodile ZOO Protivín can be seen.

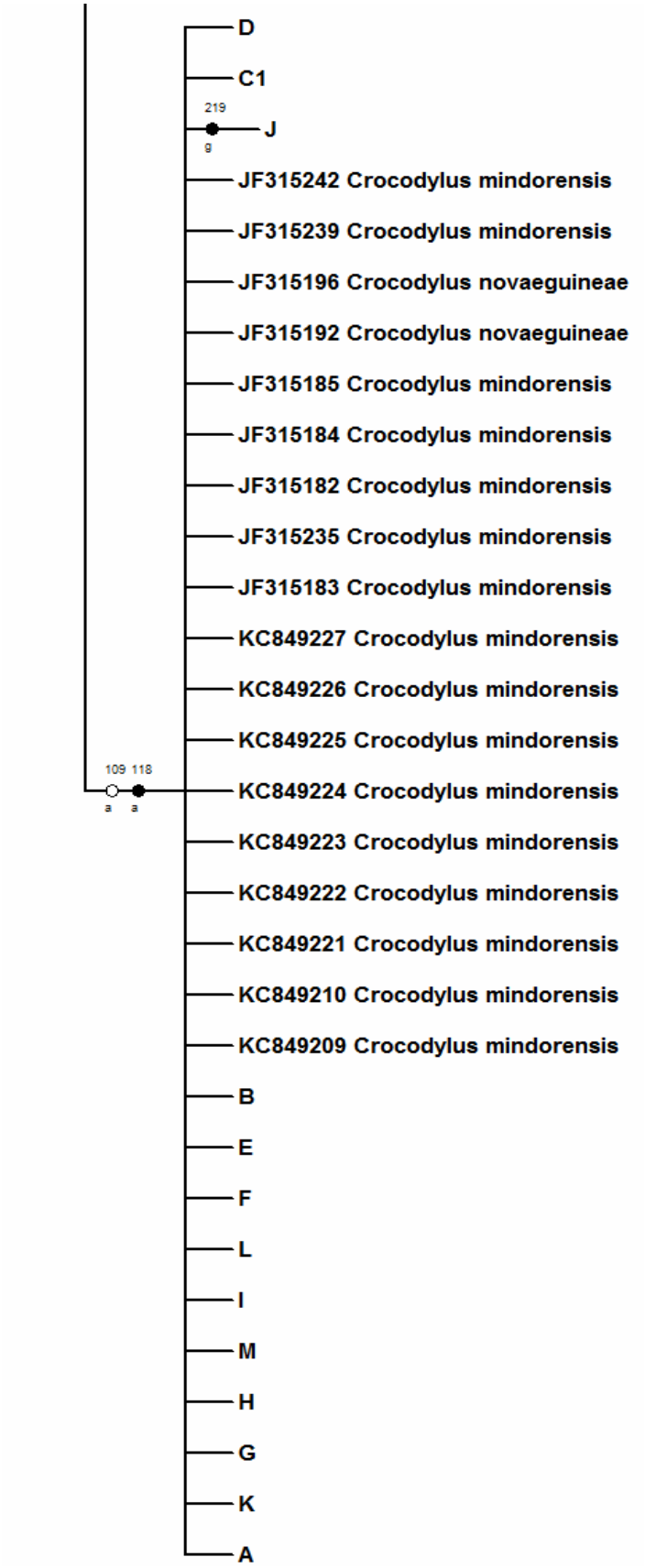

Fig. 10A. A section from the strict consensus (MP) for C-*mos* with variable sites. All *C. mindorensis* from the Crocodile ZOO Protivín can be seen.

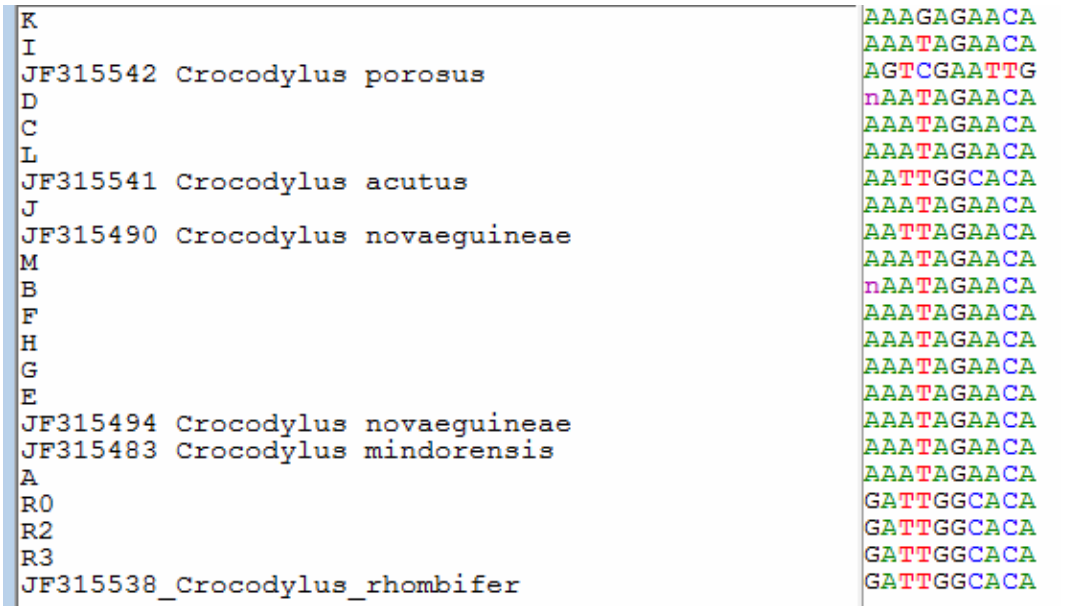

Fig. 11A. A section from alignment for LDHA in BioEdit showing variable sites in studied individuals and potentially hybridizing species.

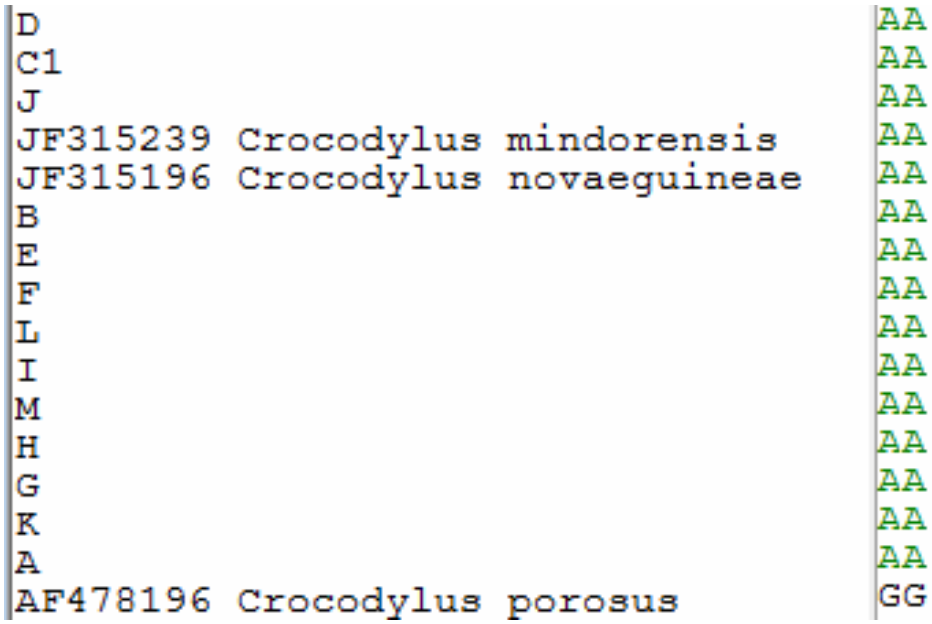

Fig. 12A. A section from alignment for C-*mos* in BioEdit showing variable sites in studied individuals and potentially hybridizing species.