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An unexpected occurrence - a case study on an intergeneric hybrid in giant snakes

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An unexpected occurrence - a case study on an intergeneric hybrid in giant snakes. - In recent years an increasing number of studies have identified cases of interspecific hybrids in reptiles, but intergeneric hybridisation, especially in snakes, is still only rarely known. In the current study we used several methods, SEM recordings, morphometrics, and both mitochondrial and nuclear gene analyses, to identify and analyse an intergeneric hybrid as a representative case study for the challenges related to this phenomenon. We here present evidence of intergeneric hybridisation between species of two well-studied boid genera: *Eunectes* (*E. notaeus*) and *Boa* (*B. constrictor*). For the intergeneric hybrid specimen the nuclear gene analyses result in its intermediate and separate phylogenetic position whereas morphological analyses clearly show that only some characteristics are intermediate, while other characters can be clearly assigned to either one of the parental species. The indistinct morphological character states and the conflicting phylogenetic position based on the genetic data show that such a hybrid can be extremely difficult to identify in situ and furthermore, those results can lead to false assumptions about the real identity and recognition of hybrids, e.g. when modern barcoding methods are used for fast and easy taxon-identification. Therefore, better recognition, identification and long term observations of both interspecific and intergeneric hybrids are needed to properly assess and preserve the current biodiversity.

Un événement inattendu - étude d'un cas d'hybridation intergénérique de serpents géants. - Récemment, un nombre croissant d'études ont permis d'identifier des hybridations interspécifiques chez les reptiles, mais les cas d'hybridation intergénériques demeurent rares, tout particulièrement chez

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les serpents. Dans notre étude, nous utilisons plusieurs méthodes modernes: microscopie SEM, morphométrie et analyses génétiques des gènes mitochondriaux et nucléaires, afin d'identifier et d'analyser un hybride intergénérique qui permettra de soulever les problématiques scientifiques liées à ce type d'hybridation. Nous présentons ici des arguments en faveur d'un cas d'hybridation intergénérique entre deux genres néotropicaux bien connus: *Eunectes* (*E. notaeus*) et *Boa* (*B. constrictor*). Les résultats de l'analyse des gènes nucléaires placent ce spécimen hybride intergénérique dans une position intermédiaire entre ses parents mais distincte phylogénétiquement alors que l'analyse morphologique montre clairement que seuls certains caractères sont intermédiaires, alors que d'autres peuvent être clairement assignés à l'une ou l'autre des deux espèces parentales. Les caractères morphologiques non diagnostics d'un taxon connu et la position phylogénétique conflictuelle obtenue par les données génétiques montre que ce type d'hybride intergénérique peut se révéler extrêmement difficile à identifier *in situ*. Une identification erronée est alors fortement probable plutôt que la détection de la nature hybride du spécimen, surtout lorsque les méthodes modernes de barcoding seront utilisées pour des identifications faciles et rapides. De ce fait, une meilleure connaissance et un suivi à long terme de tous les hybrides à la fois interspécifiques et intergénériques sera nécessaire afin d'identifier correctement la biodiversité actuelle pour appréhender sa conservation avec plus d'efficacité.

Keywords: Barcoding - BDNF - *Boa constrictor* - *Eunectes notaeus* - hybridisation - mtDNA - phylogeny - RAG1 - SEM - speciation.

INTRODUCTION

Interspecific hybrids are well known in amphibians and reptiles, but have until recently been considered as uncommon (Mertens, 1950, 1956, 1964, 1968, 1972; Murphy & Crabtree, 1988; Leaché & Cole, 2007; Mebert, 2008; Kearney *et al.*, 2009). Such interspecific hybridisation arises not only in captivity like in zoos, but also in situ where under certain circumstances hybrid zones between two distinct species occur. Especially in recent years quite a few reptile examples have been observed, e.g. in turtles [*Cuora mouhotii* x *C. galbinifrons* (Shi *et al.*, 2005), *Mauremys reevesii* x *M. sinensis* (Fong & Chen, 2010)], in different lizard families [*Anolis polylepis* x *A. osa* (Köhler *et al.*, 2010), *Aspidoscelis dixoni* x *A. tigris* (Cole *et al.*, 2007), *Podarcis siculus* x *P. waglerianus* (Capula, 1993)], in colubrids [*Pantherophis bairdi* x *P. obsoletus lindheimeri* (Vandewege *et al.*, 2012)], in vipers [*Bitis gabonica* x *B. arietans* (Broadley & Parker, 1976; Broadley, 2006)], in boids [*Eunectes murinus* x *E. notaeus* (Dirksen & Böhme, 1998)], and in pythonids [*Python natalensis* x *P. bivittatus* (Branch & Erasmus, 1984)].

While interspecific hybrids now seem not too uncommon, intergeneric hybrids, as are known between snake genera like *Liasis mackloti* x *Morelia spilota* (Banks & Schwaner, 1984) and *Crotalus horridus* x *Sistrurus catenatus* (Bailey, 1942) are apparently still very rare occurrences. One of the most recently reported occurrences of intergeneric hybridisation are two hybrid specimens of *Pituophis catenifer sayi* and *Pantherophis vulpinus* (LeClere *et al.*, 2012) which are of particular interest since these

are true naturally occurring intergeneric hybrid snakes. In the pet trade intergeneric snake hybrids are well known and some reptile breeders attempt to hybridise specific snake genera, e.g. *Pantherophis* x *Pituophis*, *Pantherophis* x *Lampropeltis*, or *Acrantophis* x *Boa* (LeClere *et al.*, 2012; Branson's Wild World, 2014; Hybrid Herps, 2014). Although several fora exist where breeders exchange their experiences, unfortunately no substantial studies exist which summarise the number of successful hybridisations in captivity and compare them to the number of known natural hybrids. Thus, one can only state that interspecific and intergeneric snakes are far better known and much more common in captivity than in nature.

Here we report on a new case of an intergeneric hybrid snake which was born in captivity and is kept in the 'Ménagerie du Jardin des Plantes', at the Paris Natural History Museum (MNHN). This living specimen is a boid hybrid between a female *Boa constrictor* and a male *Eunectes notaeus*. With the idea to shorten the phrase "intergeneric hybrid specimen" and to reflect the identity of this hybrid we name it "BOACONDA" – a joined name between the names Boa (*Boa*) and Anaconda (*Eunectes*).

Both boid genera *Eunectes* and *Boa* have been well studied (e.g. Dirksen & Böhme, 1998; Dirksen, 2002; Bertona & Chiaraviglio, 2003; Burbrink, 2005; Aller *et al.*, 2006; Bonny, 2007; Reed & Rodda, 2009) and the phylogenetic position of both genera among boid snakes has been clearly resolved in recent multigene (mitochondrial and nuclear genes) phylogenetic studies (e.g. Vences *et al.*, 2001; Burbrink, 2005; Noonan & Chippindale, 2006; Reynolds *et al.*, 2014).

The genus *Eunectes* consists of five acknowledged species and the genus *Boa* is currently believed to harbour a single species with nine subspecies. The main habitat of *Eunectes notaeus* is alongside the Rio Paraguay and its tributaries, which are part of the Pantanal. These rivers cross Bolivia, Brazil, Paraguay, Argentina and partly Uruguay (Stimson, 1969; Petzold, 1982; Henderson *et al.*, 1995; Dirksen & Böhme, 1998; Dirksen, 2002) (Fig. 1, distribution range of *Eunectes notaeus* marked with transverse lines). *E. notaeus* inhabits mainly swamps and seasonal flooded areas but it can also be found in forested or deforested as well as agricultural areas (Strüssmann & Sazima, 1993; Dirksen & Henderson, 2002; Reed & Rodda, 2009).

Boa constrictor is distributed in Central America and north and central regions of South America, from Mexico to Argentina and southern Brazil (Bonny, 2007; Reed & Rodda, 2009) (Fig. 1, distribution range of *Boa constrictor* marked with vertical lines). The species inhabits a wide range of biotopes where it is common in forests, grasslands and agricultural areas (Bonny, 2007; Reed & Rodda, 2009).

Both species *Eunectes notaeus* and *Boa constrictor* are syntopic in the northern part of the Pantanal (western Brazil) and along the upper river section of the Rio Guaporé in Bolivia (Strüssmann & Sazima, 1993; Junk *et al.*, 2006; Souza *et al.*, 2010). They prefer dense vegetation near water (Chiaraviglio, 2006; Reed & Rodda, 2009).

The hybrid BOACONDA was born on 29th May, 2009 in the "Ménagerie" of the MNHN in Paris. This snake is the only surviving individual of a clutch comprising two individuals without the skeleton, one congenital malformation and about 20 unfertilised eggs. It was sexed twice with a testing probe and identified as a male on 14th April, 2010 and 3rd December, 2011 respectively. Because of the young age of the hybrid individual sexual activity could not yet be observed, therefore, the question

FIG. 1

Distribution map: vertical lines – distribution of *Boa constrictor* spp.; transverse lines – distribution of *Eunectes notaeus*; crossed markings – overlapping distribution range of both species [modified from figures 7.2 and 8.2 of REED & RODDA (2009)].

about fertility or sterility cannot be satisfyingly answered. The BOACONDA (Figs 2 E-H), its mother (Figs 2 A-B) and both potential fathers (Figs 2 C-D) are still alive and therefore electronically tagged and their respective tag numbers are:

250228500004090, 250228700001763, 2502296000049768, and 00-01FO-7C39. The female *B. c. constrictor* arrived at the Ménagerie on 28th September, 2005 and she was previously never in contact with any male snake (I. Ineich, pers. comm.). Since the arrival day the female *B. c. constrictor* is kept in the same terrarium as the two male *E. notaeus*. Copulation was observed several times by snake keepers at the Ménagerie in 2007 and 2008.

MATERIAL AND METHODS

Genetic analyses

To determine the respective position of the hybrid in phylogenies calculated on the basis of different commonly used gene sequences (both mitochondrial and nuclear genes), we used tissue samples (obtained through biopsies) from the hybrid as well as its biological mother (*B. c. constrictor*) and both of the potential paternal individuals (*E. notaeus*). DNA was extracted from each tissue sample using peqGold Tissue DNA Mini Kit (PEQLAB). A fragment of the mitochondrial 16S rRNA gene was amplified with the primers 16sar-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16sbr-H (5'-CCGGTCTGAACTCAGATCACGT-3') (Palumbi *et al.*, 2002). Furthermore, two nuclear genes were amplified: a part of the RAG1 gene using the primers RAG1MartFL1 (5'-AGCTGCAGYCARTAYCAYAARATGTA-3') and RAG1AM-PR1 (5'-AACTCAGCTGCATTKCCAATRTCA-3') of Chiari *et al.* (2004) and a fragment of the BNDF gene using the primers BDNF-F (5'-GACCATCCTTTTCCTK-ACTATGGTTATTTCATACTT-3') and BDNF-R (5'-CTATCTTCCCCTTTTAATG-GTCAGTGTACAAAC-3') of Noonan & Chippindale (2006). We used the amplification protocols described in Chiari *et al.* (2004), Schmitz *et al.* (2005a), and Crottini *et al.* (2009) for 16S, RAG1 and BDNF, respectively. The PCR products were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH) in accordance with the manufacturer's instructions. For quality assurance both directions of the amplified PCR product were sequenced by an external vendor (Macrogen). New sequences were generated for five *Boa constrictor*, one *Calabaria reinhardtii*, two *Eunectes notaeus* and the hybrid (Boaconda). Accession numbers for the newly generated sequences are shown in the Appendix I.

Complementary sequence data for the completion of our datasets for the respective phylogenetic analyses were obtained from GenBank (see Appendix I).

The obtained sequences were initially automatically aligned using ClustalW (Thompson *et al.*, 1994) and manually checked using the original chromatograph data in the program BioEdit (Hall, 1999).

We used neighbour-joining (NJ), maximum likelihood (ML) and Bayesian interference methods to calculate the phylogenetic trees for the respective genes. NJ analyses was performed using PAUP* 4.0b10 (Swofford, 2002). For the ML tree we used the PhyML 3.0 computer cluster of the Montpellier bioinformatics platform (http://www.atgc-montpellier.fr/phyml/) (Guindon *et al.*, 2010). Bootstrap analysis (20000 [for NJ] and 2000 [for ML] pseudo-replicates) was used to estimate node support. Bayesian reconstructions were performed with MrBayes, version 3.12 (Huelsenbeck & Ronquist, 2001). Estimation of the correct parameters for the both the

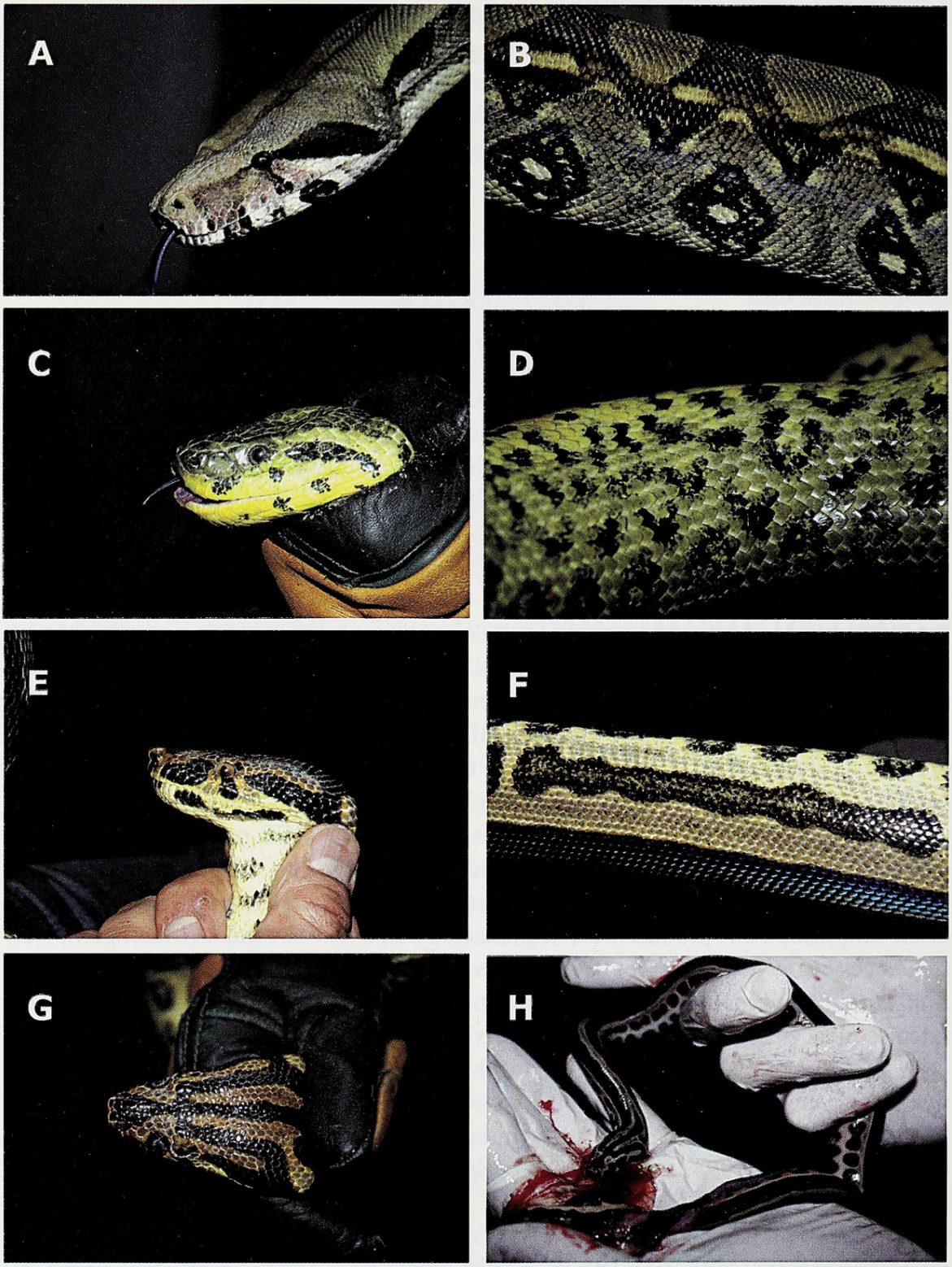

FIG. 2

(A, B) *Boa c. constrictor* (250228700001763). (C, D) *Eunectes notaeus* (2502296000049768), the specimen 0001FO7C39 is similar in colouration as the other *E. notaeus*. (E-H) BOACONDA (250228500004090), with (H) shortly after birth.

Bayesian and the ML analyses were done using jModelTest (Guindon & Gascuel, 2003; Posada, 2008). The exact parameters used for the Bayesian analyses followed those described in detail by Reeder (2003) and Schmitz *et al.* (2005b). Node support of bootstraps ≥70 % (Hillis & Bull, 1993) and Bayesian posterior probabilities ≥0.95 were considered to be highly significantly supported.

Scale microstructure

For the SEM (Scanning Electron Microscope) recordings, dried exuviae from both parent species (3 potential species: 2 paternal *Eunectes notaeus* and 1 maternal *Boa c. constrictor* and the hybrid were used. The microstructure of snake scales is unique among different species and shows almost no variation between individuals of one species and furthermore, it is independent of the individual age (N. Ernst unpubl. data; Schmidt & Gorb, 2012). Therefore, only one of the *Eunectes notaeus* individuals (00-01FO-7C39) will be described in detail. The samples from each body side (dorsal and ventral) were attached to a standard pin stub mount with a double sided carbon adhesive tape. The samples were powdered with a layer of 50 nm gold-palladium composite using a Hummer VII sputtering system (Anatech LTD, Alexandria, VA) with a 120 m Torr vacuum.

The observations were done with a HITACHI S-2460N Natural Scanning Electron Microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 25 kV and pictures were electronically displayed with the Digital Image Scanning System 5 (Version 5.4.14.2, copyright 2004) and exported to the Digital Image Processing System 2.6 (Version 2.6.14.1, copyright 1997-2005) by which the pictures were saved as JPEG and TIFF files. Microstructures of the anterior, middle and posterior regions of both dorsal and ventral scales were examined. Images of the hinge region (part of skin between scales) were also taken. These were taken at a magnification of 2.000x and 6.000x. The primary microstructure can be seen in the middle region of a scale.

Pholidosis and morphometrics

We selected three body and two head scale counts, four body and seven head measurements for the morphological analysis. Additionally, the gender and the eye-colour (EYC) (only of the four living specimens) were recorded (Table 3). Ventral (VEN) and subcaudal (SUC) scale numbers were counted according to standard techniques, as were the dorsal scale rows at midbody (DOR) (Dowling, 1951). The numbers of the supralabial (SUL) and of the infralabial scales (IFL) were counted. Following head measurements were taken with an digital calliper (Brüder Mannesmann Werkzeuge, Remscheid, Germany): the head length, which was measured from the posterior end of the lower jaw bone to the snout end (HEL); the head width, which was measured as the distance between the mandibular joints (HWI); the distance between the eyes, measured dorsally (DSE); maximal eye diameter (EYD); the distance between the nares, measured dorsally (DNA); maximum dorso-ventral diameter (DIH); maximum lateral diameter (DIW). Additionally, the snout-vent length (SVL) and the tail length (TAL) were taken with an inextensible strap and measured with a folding meter stick. The total length (TOL) was calculated by adding up the snout-vent length (SVL) and the tail length (TAL). For the analysis we calcu-

lated some ratios (TAL/TOL; HEW/HEL; HEL/SVL; HEL/TOL; DSE/HEW; DSE/HEL; EYD/HEL; DNA/HEW; DNA/HEL; DIW/DIH). All measurements were taken on the right side of the snakes. We measured the BOACONDA, the mother (*Boa c. constrictor*), the two potential fathers (*Eunectes notaeus*, 2502296000049768 and 00-01FO-7C39), and seven museum specimens of *E. notaeus* and eight museum specimens of *B. c. constrictor* from the Natural History Museum of Geneva, Switzerland (MHNG) (see Appendix II). Additionally the weight (WEI) was recorded, the coloration described and the eye colour (EYC) of the four living specimens were determined. The eye colour was described with the colour catalogue for field biologists by Köhler (2012).The statistical analyses [Univariate Analysis, Principal Component Analysis (PCA), with variances and covariances of groups, and between-group calculations] were conducted using PAST version 2.16 (Hammer *et al.*, 2001).

RESULTS

GENETIC ANALYSES

Of the three computed phylogenetic gene trees (Figs 3 A-C), the mitochondrial tree shows as expected a complete sequence identity of the BOACONDA with its maternal lineage (*Boa c. constrictor*) and thus both the confirmed mother and the hybrid offspring are placed in the same well supported clade. In contrast to the mitochondrial tree, the hybrid is placed in an approximately intermediate position between its parental species *Eunectes notaeus* (2502296000049768, 00-01FO-7C39) and *Boa c. constrictor* in both computed trees for the nuclear genes, even though contrarily to ML the MrBayes package treats heterozygous (ambiguous) sites as missing data (Potts *et al.*, 2014). The nuclear genes used do not allow us to determine which one of the male *E. notaeus* individuals is the actual father, but since there were absolutely no differences in both nuclear genes between the two *E. notaeus* specimens, we treat both specimens equally.

The two parental genera are situated on highly significantly supported distinct clades and are well separated from each other. Both the BDNF- and RAG1-tree (Figs 3 B-C) show that the integration of hybrids does not significantly alter the node support for the parental taxa. The intermediate position can be explained due to heterozygosity at most or all of the 12 variable sites in the BDNF gene fragment and 19 variable sites in the RAG1 gene fragment. 11 of the variable sites (12) in the BNDF gene fragment between *B. c. constrictor* mother and *E. notaeus* potential fathers are identified as fixed synapomorphies (Table 1) and all 19 variable sites in the RAG1 gene fragment are synapomorphies in *B. c. constrictor* and *E. notaeus* (respectively 2502296000049768 and 00-01FO-7C39) (Table 2). The hybrid shows heterozygosity at 83 % of variable sites in the BDNF-gene fragment and 100 % of variable sites in the RAG1-gene fragment.

SCALE MICROSTRUCTURE

The microstructure of the dorsal scale (Fig. 4 A) of *Boa c. constrictor* shows cells which are irregularly shaped and mostly longer than they are wide. The cell borders are primarily smooth and form anterior a few elongated, broad peaks. The pores of the cells are elongated, almost regularly aligned, touch the cell borders, and

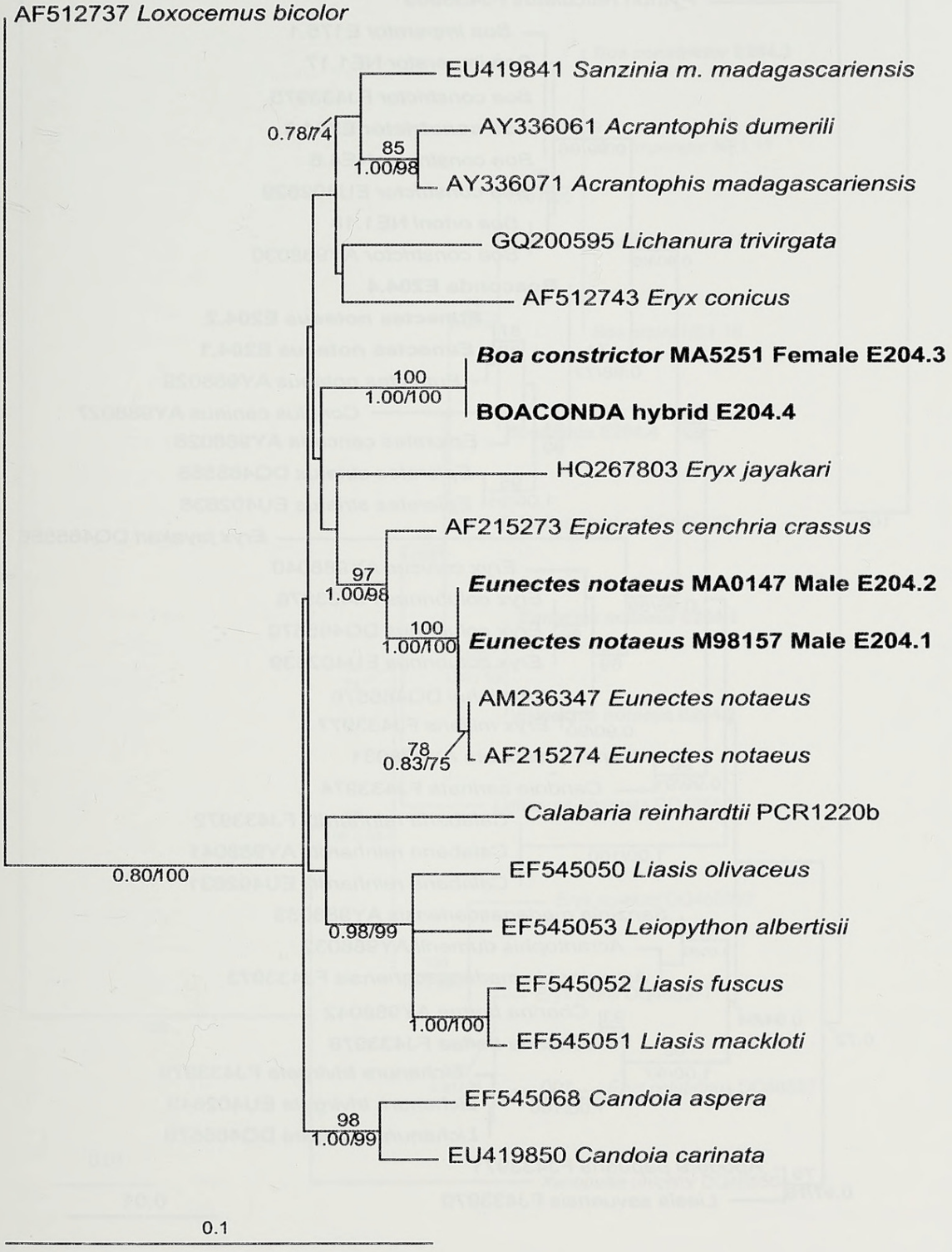

FIG. 3A

Phylogenetic tree based on the mitochondrial gene fragment 16S with calculated node support for ML analysis above the branches (only node supports over 70% are listed), and Bayesian analysis (only node supports over 0.70 are listed) and calculated NJ node support under the branches (only node supports over 60 % are listed).

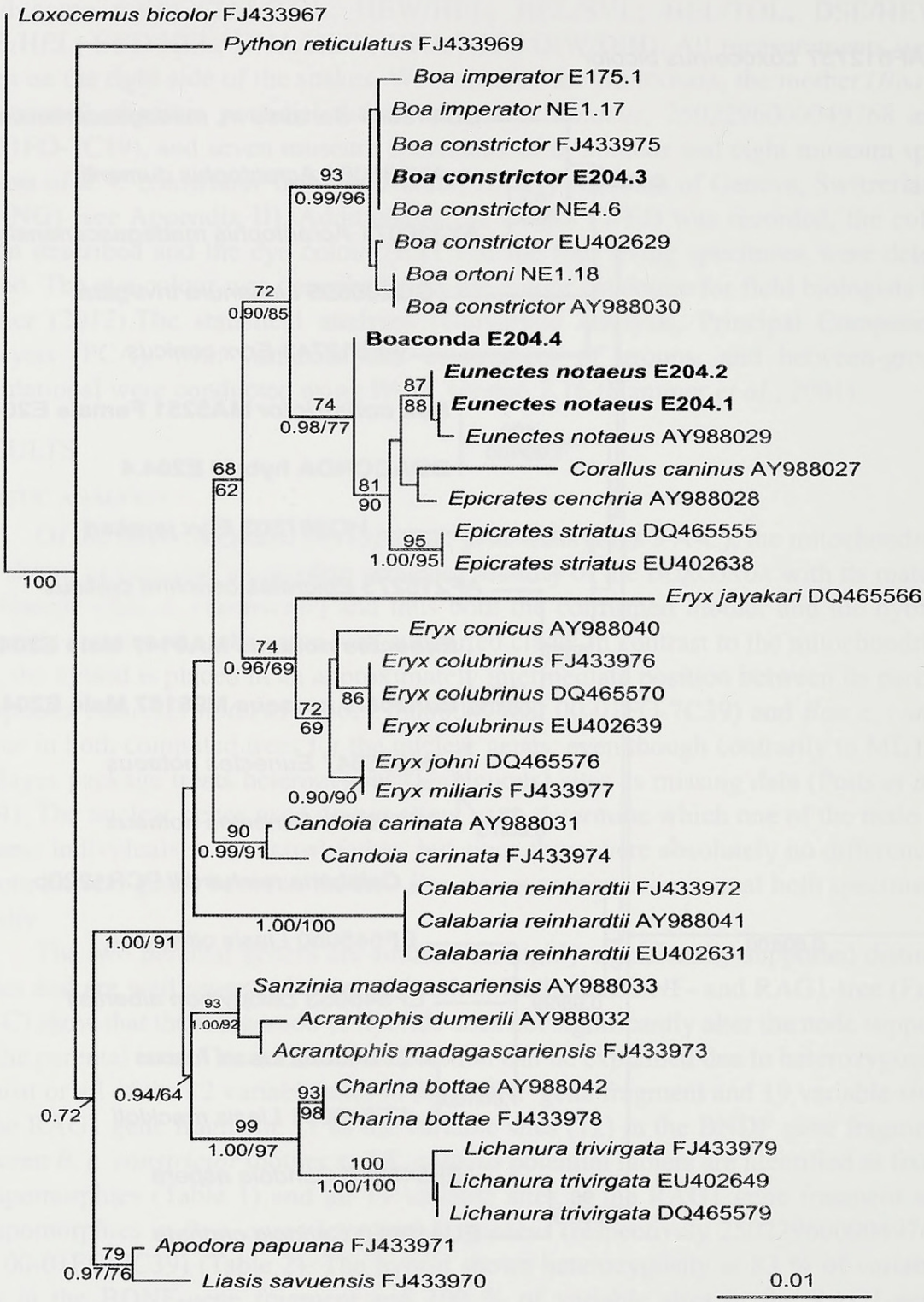

FIG. 3B

Phylogenetic tree based on the nuclear gene fragment BDNF with calculated node support for ML analysis above the branches (only node supports over 70% are listed), and Bayesian analysis (only node supports over 0.70 are listed) and calculated NJ node support under the branches (only node supports over 60 % are listed).

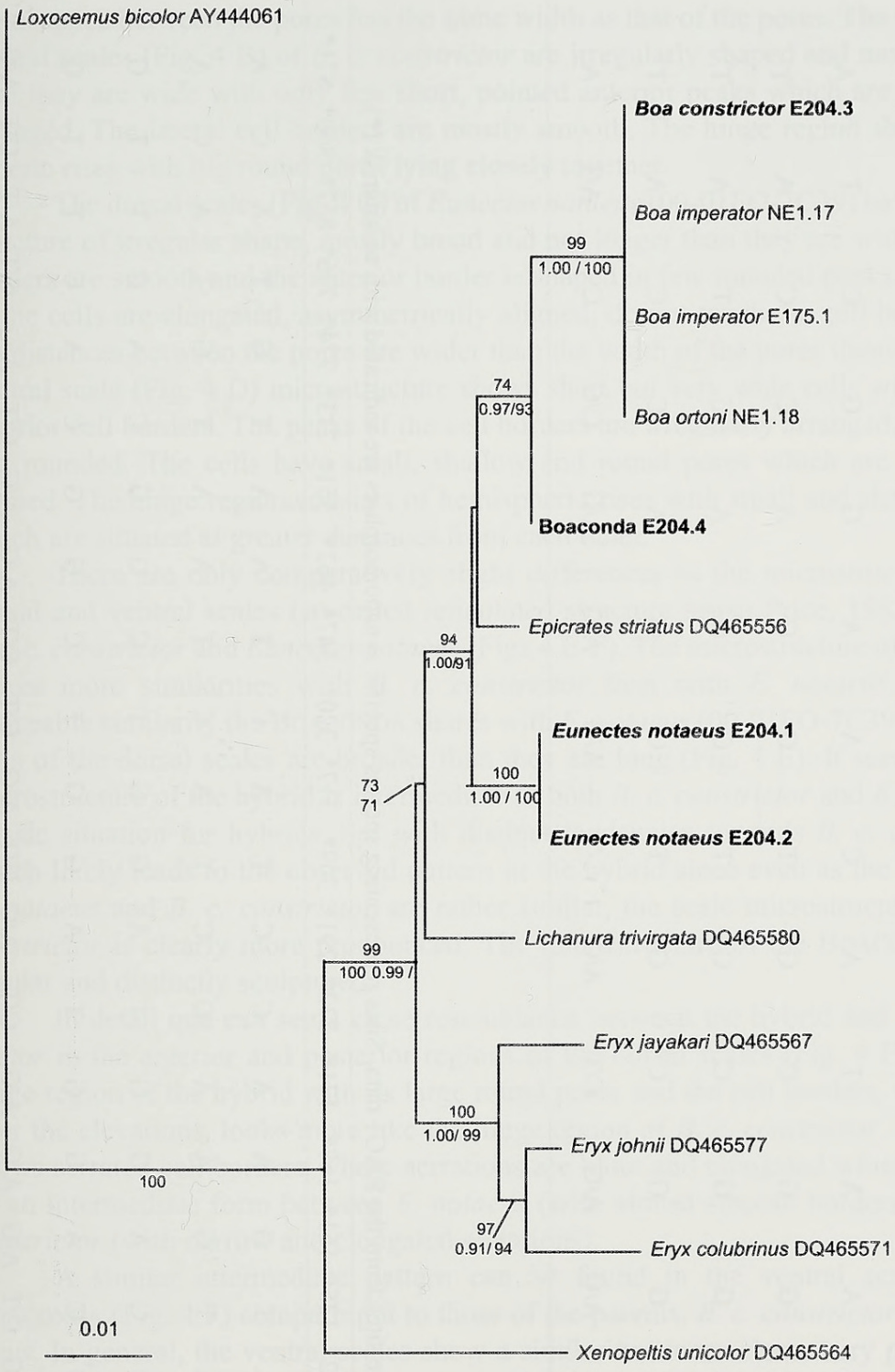

FIG. 3C

Phylogenetic tree based on the nuclear gene fragment RAG1 with calculated node support for ML analysis above the branches (only node supports over 70% are listed), and Bayesian analysis (only node supports over 0.70 are listed) and calculated NJ node support under the branches (only node supports over 60 % are listed).

TABLE 1: Variable sites from the BDNF data. Sites representing species specific synapomorphies are indicated with an asterisk (*)

Sample	123*	209*	251*	263*	267*	356*	392*	470*	497*	647*	659*	710*
Eunectes notaeus (0001FO7C39)	A	A	T	A	C	A	A	G	G	C	T	A
Eunectes notaeus (2502296000049768)	A	A	T	A	C	A	A	G	G	C	T	A
Boa c. constrictor (250228700001763)	G	G	C	G	T	G	G	A	C	C	A	T
Boa c. constrictor (NE4.5)	G	G	C	G	T	G	G	A	C	T	A	T
Boa c. imperator (E175.1)	G	G	C	G	T	G	G	A	C	C	A	T
BOACONDA (250228500004090)	A/G	A/G	C/T	A/G	C/T	A/G	A/G	A/G	C/G	C	A/T	A

TABLE 2: Variable sites from the RAG1 data. Sites representing species specific synapomorphies are indicated with an asterisk (*).

Sample	122*	125*	161*	167*	171*	232*	291*	309*	317*	320*	347*	462*	551*	713*	774*	776*	857*	860*	871*
Eunectes notaeus (0001FO7C39)	A	T	G	A	A	C	C	T	A	C	T	A	A	T	G	T	A	C	A
Eunectes notaeus (2502296000049768)	A	T	G	A	A	C	C	T	A	C	T	A	A	T	G	T	A	C	A
Boa c. constrictor (250228700001763)	G	C	A	G	G	T	A	G	G	T	C	G	G	C	A	C	T	T	G
Boa c. imperator (175.1)	G	C	A	G	G	T	A	G	G	T	C	G	G	C	A	C	T	T	G
BOACONDA (250228500004090)	A/G	C/T	A/G	A/G	A/G	C/T	A/C	G/T	A/G	C/T	C/T	A/G	A/G	C/T	A/G	C/T	A/T	C/T	A/G

the distance between the pores has the same width as that of the pores. The cells of the ventral scales (Fig. 4 B) of *B. c. constrictor* are irregularly shaped and mostly longer than they are wide with only few short, pointed anterior peaks which are irregularly arranged. The lateral cell borders are mostly smooth. The hinge region shows hemispheric rises with big round pores lying closely together.

The dorsal scales (Fig. 4 C) of *Eunectes notaeus* (00-01FO-7C39) have a microstructure of irregular shape, mostly broad and not longer than they are wide. The cell borders are smooth and the anterior border is shaped in few rounded peaks. The pores of the cells are elongated, asymmetrically aligned, do not touch the cell borders, and the distances between the pores are wider than the width of the pores themselves. The ventral scale (Fig. 4 D) microstructure shows short but very wide cells with serrated anterior cell borders. The peaks of the cell borders are irregularly arranged, very short and rounded. The cells have small, shallow and round pores which are irregularly aligned. The hinge region consists of hemispheric rises with small and shallow pores which are situated at greater distances from each other.

There are only comparatively slight differences in the microstructure of the dorsal and ventral scales (so-called reticulated structure sensu Price, 1982) between *Boa c. constrictor* and *Eunectes notaeus* (Figs 4 E-F). The microstructure of the hybrid shares more similarities with *B. c. constrictor* than with *E. notaeus*. The only noticeable similarity the BOACONDA shares with *E. notaeus* (00-01FO-7C39) is that the cells of the dorsal scales are broader than they are long (Fig. 4 E). It seems that the microstructure of the hybrid is intermediate to both *B. c. constrictor* and *E. notaeus*, a classic situation for hybrids, but with distinct tendencies towards *B. c. constrictor*; which likely leads to the observed pattern in the hybrid since even as the patterns of *E. notaeus* and *B. c. constrictor* are rather similar, the scale microstructure of *B. c. constrictor* is clearly more pronounced. The microstructure of the BOACONDA looks regular and distinctly sculptured.

In detail one can see a close resemblance between the hybrid and *B. c. constrictor* in the anterior and posterior regions of the dorsal scales (Fig. 4 E). Also the hinge region of the hybrid with its large round pores and the cell borders, which span over the elevations, looks more like the hinge region of *B. c. constrictor*. The hybrid shows serrated cell borders. These serrations are blunt and elongated which appear to be an intermediate form between *E. notaeus* (with almost smooth borders) and *B. c. constrictor* (with narrow and elongated serrations).

A similar intermediate pattern can be found in the ventral scales of the BOACONDA (Fig. 4 F) comparing it to those of the parents, *B. c. constrictor* and *E. notaeus*. In general, the ventral scales show a similar but more elementary pattern than the pattern of the dorsal scales. A remarkable similarity in the microstructure can be found between *B. c. constrictor* and the hybrid with elongated ridges and punctate pores in-between, whereas *E. notaeus* has bigger rounded pores (Fig. 4 F). The primary microstructure of the BOACONDA's cell borders shows an intermediate pattern to *B. c. constrictor* and *E. notaeus* respectively. The cell borders of the hybrid are shaped in long and broad serrations, while the borders of *B. c. constrictor* are almost smooth and *E. notaeus* has cell borders which show short and narrow serrations.

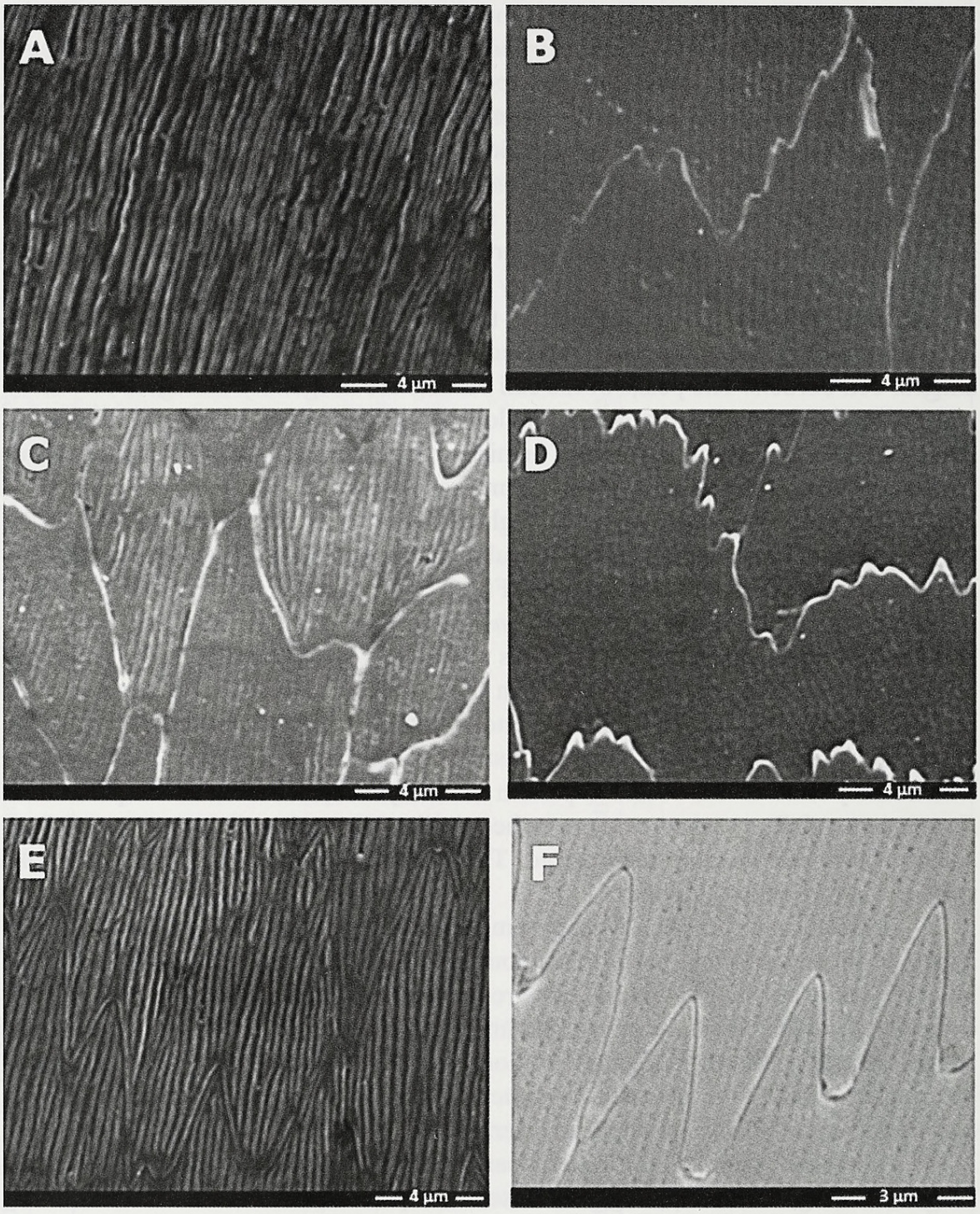

FIG. 4

SEM recordings. (A) Dorsal scale of *Boa c. constrictor* (250228700001763). (B) Ventral scale of *Boa c. constrictor* (250228700001763). (C) Dorsal scale of *Eunectes notaeus* (0001FO7C39). (D) Ventral scale of *Eunectes notaeus* (0001FO7C39). (E) Dorsal scale of BOACONDA (250228500004090). (F) Ventral scale of Boaconda (250228500004090).

PHOLIDOSIS AND MORPHOMETRICS

The pholidosis and morphometrics show an interesting pattern. The numbers of ventral scales (VEN), the ratio of tail length to the total length (TAL/TOL), the ratio of the distance between the eyes towards the head length (DSE/HEL), and the ratio of the

TABLE 3: Pholidosis and ratios of morphometrics.

	Mean value	Minimum value	Maximum value		Mean value	Minimum value	Maximum value
	Eunectes notaeus			BOACONDA	*Boa c. constrictor*		
DOR	48	45	51	57	87.22	82	95
VEN	232.67	228	242	244	246.67	236	266
SUL	13.78	13	16	18	21.11	19	24
IFL	17.33	17	18	20	24.33	22	28
TAL/TOL	15.74	11.79	19.05	12.00	11.99	10.23	13.13
HEL/TOL	3.68	3.21	4.01	3.57	4.41	3.67	5.85
DSE/HEL	26.65	24.93	29.15	35.46	35.39	33.73	39.38
DNA/HEL	12.41	11.03	16.64	16.56	14.76	13.29	17.02
EYD/HEL	8.86	7.85	10.51	9.44	7.64	6.98	8.55

distance between the nares towards the head length (DNA/HEL) of BOACONDA are in the range of the values of *Boa c. constrictor* (Table 3, Figs 5 A-B). In contrast to this, the ratio of the head length towards the total length (HEL/TOL), and the ratio of the eye diameter towards the head length (EYD/HEL) lie in the ranges of the values of *Eunectes notaeus* (Table 3, Figs 5 C-D). The numbers of the supralabial (SUL) and of the infralabial (IFL) scales are intermediary between *B. c. constrictor* and *E. notaeus* (Table 3, Fig. 5 F) as well as the count of the dorsal scale rows (DOR) (Table 3, Fig. 5 E).

COLOURATION

The colouration of the BOACONDA (Figs. 2 E-H) shows also intermediate characteristics and only few distinct characters are shared with one of the parental species. The ground-colouration is a light yellow similar to the potential fathers. Dorsally are two brown blotches many of them are fused to stripes. The female *Boa c. constrictor* (Figs. 2 A-B) shows a light brown ground colour and the typical large, dark brown saddles. Both potential fathers [*E. notaeus*, 2502296000049768 (Figs. 2 C-D), 00-01FO-7C39] are yellow-green coloured with small, black spots and the typical small, saddles. The BOACONDA has black large roundish blotches on the flanks (Figs 2 F, 2 H), while the mother (Fig. 2 B) has rhombic blotches with greater distances to each other and the potential fathers (Fig. 2 D) have small blotches. The ventral side of the mother is cream-coloured with brownish blotches in greater distance to each other and the potential fathers have small black spots on a yellow ventral side, while in contrast the BOACONDA has two rows of adjacent black blotches. The head of the Boaconda (Figs 2 E, 2 G) shows a median stripe from the top of the snout and two stripes right and left of the median stripe, which begin at eye level. Additionally, the snake has a large black blotch before and a large black stripe after the eye. The mother shows the typical small light brown central stripe and laterally a thinner stripe behind the eye (Fig. 2 A). The potential fathers have three stripes, which are only slighter darker than the yellow-green ground colour. Laterally *E. notaeus* (Fig. 2 C) has a small blotch in front of the eye and a thinner stripe behind the eye.

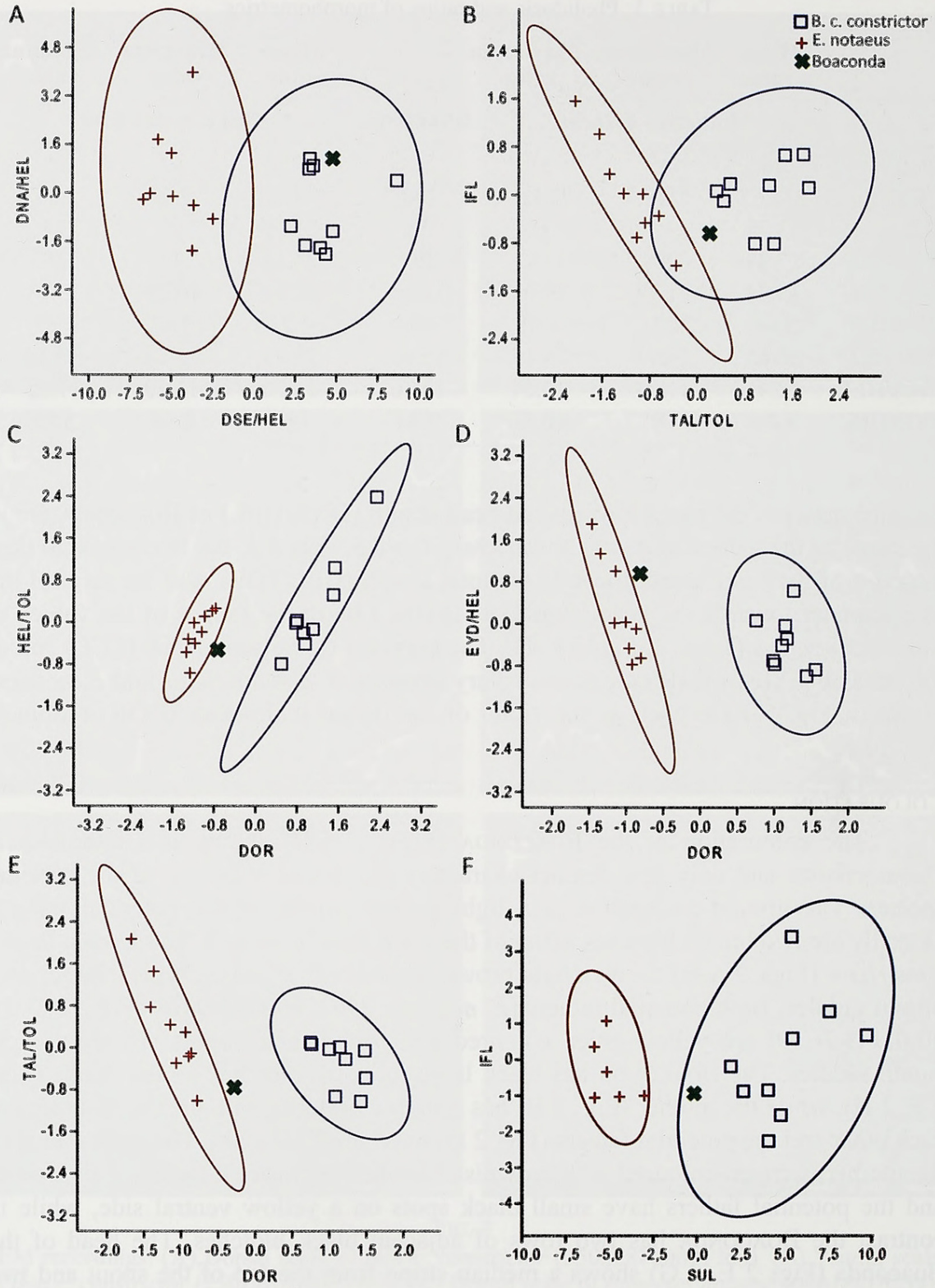

FIG. 5

Principal Component Analyses of the discussed characters (ellipses indicate estimation where 95% of the individuals of the population are expected to fall; DNA/HEL – ratio between distance of nares and head length, DOR – dorsal scale rows at midbody, DSE/HEL – ratio between distance of eyes and head length, EYD/HEL – ratio between eye diameter and head length, HEL/TOL – ratio between head length and total length, IFL – numbers of infralabial scales, SUL – numbers of supralabial scales, TAL/TOL – ratio between tail length and total length).

DISCUSSION

Hybrid individuals do not always show morphologically intermediate characteristics between the distinct characters known from their parent species (Ross & Cavender, 1981; Mebert, 2008, 2010; Toda & Hikida, 2011) but at the same time not all morphological intermediates are hybrids (Wilson, 1992; Dowling & Secor, 1997). Sometimes hybrid offspring have absolutely no detectable morphological unique characteristics but only show those characteristics which are already present in one of the parent species as recently shown in the study of Mebert (2010) about hybrid zones between the colubrid snakes *Nerodia fasciata* and *N. sipedon* and in the study of Toda & Hikida (2011) about the hybrids of the geckos *Gekko yakuensis* and *G. hokouensis*.

The SEM and morphological analyses also show that some intermediate characteristics are present but others show clear tendencies to the traits present in one of the parent species. The SEM analyses show that although there are scale microstructures of the hybrid which are intermediate between *Boa c. constrictor* and *Eunectes notaeus* (00-01FO-7C39), there is a pronounced tendency towards the typical structures observed in *B. c. constrictor*. Assuming one does not know in advance that the scales analysed are those of a hybrid specimen, at first glance, the scale microstructure could lead to the false assumption that these are the scales of a *Boa c. constrictor*. Some characters are indicative of the hybrid status, e.g. one remarkable intermediate scale microstructure is the shape of the cell borders of the dorsal and ventral scales of the hybrid. As these specific structures are not routinely analysed, such deviations in character states can be easily overlooked. Only few morphological characters of BOACONDA show clear intermediate states towards the parental species (Figs. 5 E-F), but some other characters (ventral scales, DNA/HEL, DSE/HEL) fall directly in the range of the maternal species *B. c. constrictor* (Figs. 5 A-B) and some other characters (EYD/HEL, HEL/TOL) fall in the range of the paternal species *E. notaeus* (Figs. 5 C-D). Therefore, not only the analyses of mitochondrial gene fragments of questionable hybrid specimens can lead to false assumptions, namely that the hybrid is not identified as a hybrid but is assigned to the maternal species (see discussion below), but also in-depth morphological data can lead to the assumption that such a hybrid snake specimen is wrongly identified as a member of one of the parental species.

In this case the hybrid was born in captivity. But considering the recently published paper about the *Pituophis catenifer sayi* x *Pantherophis vulpinus* hybrids which are indeed naturally occurring hybrids (LeClere *et al.*, 2012), it can be assumed that also naturally occurring hybridisation between *Eunectes notaeus* and *Boa constrictor* may be possible for several reasons, among them the fact that the geographic distribution areas of both species overlap in larger parts (see Fig. 1). Both species are sympatric in the northern part of the Pantanal (western Brazil) and along the upper river section of the Rio Guaporé in Bolivia. Additionally, *E. notaeus* and *B. constrictor* show very similar aspects regarding their reproductive biology, as well as in sexual dimorphism and mating habits. Adult females of both *E. notaeus* and *B. constrictor* are distinctly larger than the corresponding adult males. During the mating season both species form mating aggregations (Dirksen, 2002; Bertona & Chiaraviglio, 2003; Rivas & Burghardt, 2005). The mating season of *E. notaeus* is between September and

December (Dirksen, 2002) and the mating season of *B. constrictor* is during the dry season starting approximately in June and lasting until September (Bertona & Chiaraviglio, 2003; Pizzatto & Marques, 2007). Based on the similar mating habits, the overlapping mating season and the sympatric occurrence in the same habitat of both species it is quite likely that the mechanism which should prevent hybridisation can easily break down e.g. due to habitat disturbance – be it caused by climate change or human impact (Bullini, 1985; Barton & Bengtsson, 1986; Birky, 2013).

The potential high competitiveness (through heterosis) of most hybrid species can be explained by the increased enhancement of heterozygosity in a single generation where interspecific hybridisation occurs (Bullini, 1985). Grant & Grant (1994) discovered that hybrids and backcrosses of the Ground Finches *Geospiza fortis*, *G. scandens* and *G. fuliginosa* on the island Daphne Major in the Galápagos Archipelago exhibit higher fitness levels than their parental species. Furthermore, hybridisation and introgression can probably more rapidly increase genetic diversity through production of new recombinant genotypes than it is possible by mutation (Dowling & Secor, 1997) and such enhanced variability could allow organisms to expand their range in unfavourable habitats and to adapt more readily to environmental changes (Stebbins, 1959; Dowling & Secor, 1997; Martinez-Freiria *et al.*, 2010). Such an increase of the genetic diversity and adaptation to a changing environment can benefit speciation. Another possibility to establish a stable hybrid zone or population is by parthenogenesis (Murphy *et al.*, 2000; Schmitz *et al.*, 2001; Strasburg *et al.*, 2007; Bengtsson, 2009). But not only parthenogenetic stable communities can establish evolutionary isolated lineages. Recent genetic studies reveal evidence that the red wolf (*Canis rufus*) has originated from the coyote (*Canis latrans*) by historical hybridisation with the grey wolf (*Canis lupus*) (Wayne & Jenks, 1991; Roy *et al.*, 1994; von Holdt *et al.*, 2011).

All these factors discussed above may lead to a generally increased hybridisation rate in the long run, and thus to complications in efficiency of modern fast screening techniques like DNA barcoding. The main intent of the DNA barcoding is to rapidly identify unknown taxa and to facilitate the discovery of new species using large-scale screening (Hebert *et al.*, 2003; Stoeckle, 2003; Eaton *et al.*, 2010; Nagy *et al.*, 2012). For this approach mostly mitochondrial gene fragments have been used. Although intergeneric hybridisation is still mostly regarded as uncommon and thus should theoretically have only little impact on DNA barcoding (Hebert & Gregory, 2005) the increasing identification of hybrid specimens (Bullini, 1985) shows that the problems these specimens cause in barcoding screenings should not be underestimated (Eaton *et al.*, 2010). In our case study the true hybrid status of the BOACONDA was not correctly identified in any of the three phylogenies despite using different and commonly used genetic markers. While we accept the concept of DNA barcoding as a useful first screening technique, we want to draw attention to the fact that there are aspects which can easily be overseen or can lead to false assumptions even when dealing with seemingly well-known taxa. When using mitochondrial gene fragments for barcoding as currently established, you will always fail to detect a hybrid since a hybrid specimen will always be identified as an individual belonging to its maternal species (see Fig. 3 – 16S tree). But even following the current trend to use additional gene fragments, e.g. using nuclear genes to resolve the deeper nodes in a phylogeny, a

hybrid will be mostly positioned in an isolated clade which will be intermediate somewhere between the (also well supported) clades containing both parental species; thus, it could easily be considered as a new undescribed taxon. Since even phylogenetic programs like the widely used MrBayes, which handle heterozygous data as missing data (Potts *et al.*, 2014), may recover intermediate positions for hybrids in phylogenetic trees, only a direct analysis of heterozygous sites, and a specific integration into molecular datasets, e.g. using the 2ISP-informative approach (Potts *et al.*, 2014) or by phasing the nuclear gene haplotypes and analysing the alleles separately (Weisrock *et al.*, 2012), can clarify whether the specimen in question is of hybrid origin or not and properly determine its phylogenetic position..

Dubois (1981a, 1981b, 1983, 1988a, 1988b, 2004) and Dubois & Bour (2010) raised an interesting aspect concerning intergeneric hybrids. They propose that two species, which are able to produce (either under natural or artificial conditions) viable adult hybrids, should not be included in different genera. The genus as a systematic unit should be seen as a species or a group of species of presumably common phylogenetic origin which is separated by a decided gap from other similar groups (Mayr, 1942; Lemen & Freeman, 1984; Dubois, 1988). While it is clear that the allocation of taxa to genera is an artificial and subjective method to categorise these taxa, the existing data leave no doubt about the validity of both the genera *Boa* and *Eunectes*. The morphological, genetic, and ecological data known for these two genera and their closest relatives [*Epicrates* s.l. (Caribbean Islands), *Epicrates* s.l. (South America), and *Corallus* (Carribean Islands, Central and South America)] show clear separations and differentiations between each of them (Tolson, 1987; Kluge, 1989; Burbrink, 2005; Noonan & Chippindale, 2006; Lee *et al.*, 2007; Rivera *et al.*, 2011; Reynolds *et al.*, 2014; this study). Furthermore, following the "strategy of temporal banding" (Avise, 2008), the known age of the different genera also implicates that the recognition in different groups is justified. The combination of the above mentioned data leads to our working definition of the term genus: clear differentiations between several groups together with similarities between species within those groups in morphology, genetics, ecology and evolutionary time estimations indicate the uniqueness of the specific species groups. If we would adopt Dubois' proposal (1981a,b, 1983, 1988a,b, 2004) we would rate down the weight of all morphological, genetic, and ecological data, all of which implicate the differentiation between the four species groups in favour of a single criterion. To fuse all these distinct genera in one single genus would mean to lose quite a lot of information about their evolutionary diversity.

In this work we have shown that the potential problems associated with hybrid specimens should not be underestimated. We emphasise that hybrids both captive bred and naturally occurring ones are inherently a rich source of information, and while for a long time hybrids were considered as less fit or as a weakening factor for the associated species population, several new studies have shown that hybridisation is not always a negative factor but that hybridisation can even be a catalyst for speciation (Stebbins, 1959; Remington, 1968; Bullini, 1985; Wayne & Jenks, 1991; Roy *et al.*, 1994; Dowling & Secor, 1997; Seehausen, 2004; Mebert, 2008, 2010; Martinez-Freiria *et al.*, 2010). Ignoring potential hybrids can be problematic, since fast morphological and genetic screening techniques of high biodiversity areas are progressively gaining

favour. With the increasing rate of such studies, the results of those studies influence political decisions on the future of the studied regions (conservational status, clearings, etc.). When specific biological information like the occurrence of hybrid zones and the taxonomic status of the parental species are not properly identified, then those missing data can lead to decisions which may even be unfavourable for the parentals themselves. A typical case of such mistakes can be observed after the introduction of *Iguana iguana* in the West Indies where it clearly hybridises with the endemic *Iguana delicatissima* leading to the extinction of the latter on some islands (Breuil, 2002), but these hybrids have only been recently recognised. A similar situation can be found on the island Utila where the endemic iguanid lizard *Ctenosaura bakeri* is threatened by the hybridisation with the widespread *Ctenosaura similis* (Pasachnik *et al.*, 2009).

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APPENDIX I

The used GenBank accession numbers are as follows: for 16S: AF215273, AF215274, AF512737, AF512743, AM236347, AY336061, AY336071, EF545050, EF545051, EF545052, EF545053, EF545068, EU419841, EU419850, GQ200595, HQ267803; for BDNF: AY988027, AY988028, AY988029, AY988030, AY988031, AY988032, AY988033, AY988040, AY988041, AY988042, DQ465555, DQ465566, DQ465570, DQ465576, DQ465579, EU402629, EU402631, EU402638, EU402639, EU402649, FJ433967, FJ433969, FJ433970, FJ433971, FJ433972, FJ433973, FJ433974, FJ433975, FJ433976, FJ433977, FJ433978, FJ433979; for RAG1: AY444061, DQ465556, DQ465564, DQ465567, DQ465577, DQ465571, DQ465580.

GenBank accession numbers for the newly generated sequences are as follows: BOACONDA (250228500004090; 16S: KF576911, BDNF: KF576915; RAG1: KF576748); *Boa c. constrictor* (250228700001763; 16S: KF576910, BDNF: KF576914; RAG1: KF576751); *Boa c. constrictor* (NE4.5; BDNF: KF576787); *Boa c. imperator* (E175.1; BDNF: KF576816; RAG1: KF576905); *Boa c. imperator* (NE1.17; BDNF: KF576812; RAG1: KF576901); *Boa c. ortonii* (NE1.18; BDNF: KF576811; RAG1: KF576900); *Calabaria reinhardtii* (NE2.2; 16S: KF576930); *Eunectes notaeus* (0001FO7C39; 16S: KF576912, BDNF: KF576916; RAG1: KF576749); *Eunectes notaeus* (2502296000049768; 16S: KF576913, BDNF: KF576917; RAG1: KF576750).

APPENDIX II

Morphological data were obtained from following specimens of the collection of the Natural History Museum of Geneva (MHNG). *Eunectes notaeus* specimens: MHNG 1348.17; MHNG 1501.06; MHNG 1501.67; MHNG 1551.82; MHNG 2194.3; MHNG 2424.33; MHNG 2424.34; and *Boa c. constrictor* specimens: MHNG 12.34; MHNG 1325.33; MHNG 1337.37; MHNG 1456.83; MHNG 2238.13; MHNG 2424.44; MHNG 2424.45; MHNG 2424.46.