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RESEARCH ARTICLE

Conservation genetics of Boelen's python (*Morelia boeleni*) from New Guinea: reduced genetic diversity and divergence of captive and wild animals

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Abstract Boelen's python (*Morelia boeleni*) is a montane New Guinea endemic found in highlands above 1000 m and below the tree line. The ecology, natural history, distribution, population size, and conservation status of this species are largely unknown. It has a protected status in Papua New Guinea but not in Indonesian Papua and several US and European zoos have active captive breeding programs that have been largely unsuccessful. To understand the degree of genetic diversity in wild and captive animals we undertook a genetic analysis of 90 *M. boeleni* for which we sequenced two mtDNA loci and one nuclear locus for a total of 1,418 bp of sequence data per individual. All 16 wild-caught *M. boeleni* from Indonesia and all captive *M. boeleni* are genetically uniform for all three loci. The single wild-caught animal from Papua New Guinea showed extremely low levels of genetic divergence and diversity from the Indonesian and captive samples. Data from two congeners, *M. amethistina* and *M. viridis*, suggests that *M. boeleni* have reduced genetic variation with a small effective population size possibly due to historical bottlenecks. These data demonstrate the need for further studies of genetic diversity of *M. boeleni* from across its range and raise particular concern for the limited genetic diversity of *M. boeleni* used captive breeding programs in zoological parks.

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Introduction

The tropical island of New Guinea has been identified as one of the world's five High Biodiversity Wilderness Areas (Mittermeier et al. 2003). New Guinea occupies less than 0.6% of global land area yet harbors 5–7% of the world's biodiversity (Beehler 1993; Dinerstein and Wikramanyake 1993; Myers et al. 2000). The herpetofauna of New Guinea currently accounts for approximately 5% of the world's reptile and amphibian diversity yet surprisingly this is an underestimate of true diversity; species accumulation curves for both lizards and frogs continue to increase dramatically without hint of plateau (Austin et al. 2008). A defining feature of the island is the 2,500 km long central cordillera that forms the central highlands. The tallest peak exceeds 5,000 m and many other peaks exceed 3,000 m. The rugged highlands regions are geologically young (<5 mya) and the rugged montane topography encompasses a wide range of habitat types with a highly endemic fauna and flora (Hill and Gleadow 1989; Davies 1990; Audley-Charles 1991; Crowhurst et al. 1996).

A fairly recent discovery to science, Boelen's Python (*Morelia boeleni*), is an uncommon snake found only in the New Guinea highlands above 1000 m and below the tree line (Brongersma 1953; O'Shea 1996). In Papua New Guinea, an independent country in the eastern half of the island (Figs. 1, 2), *M. boeleni* is a protected species and is afforded the same stringent legal status as birds of paradise (O'Shea 1996). In the western half of the island, the Indonesian province of Papua, *M. boeleni* has no country specific protection although both Indonesia and Papua New

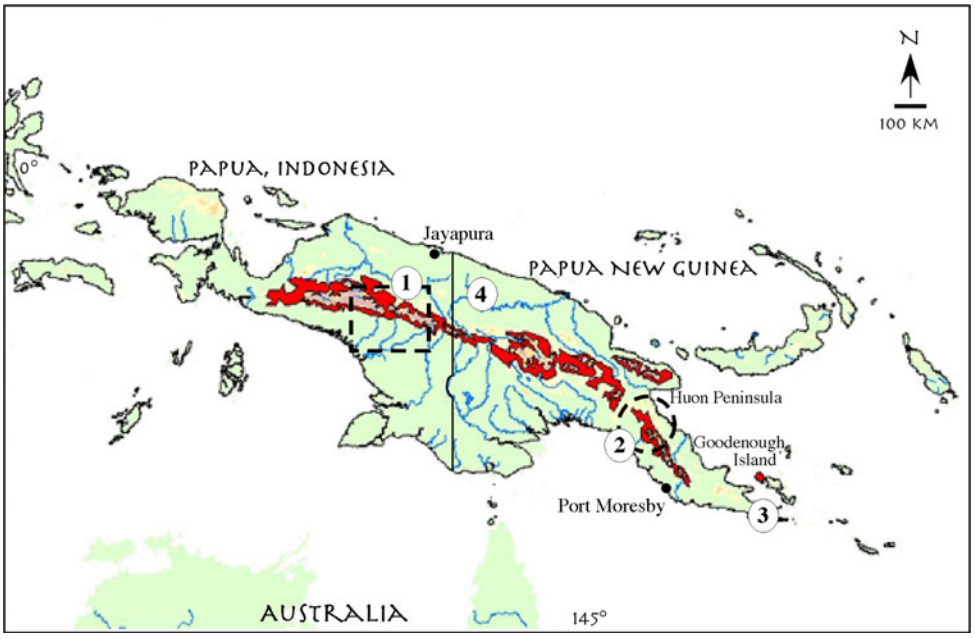

Fig. 1 Map of the approximate distribution of *M. boeleni* along the central cordillera (in *red* from above 1000 m to the *tree line*) and disjunct distributions in the Huon Peninsula and Goodenough Island based on museum records and elevation (redrawn and modified with permission from the Bishop Museum Papuan database http://www.bishopmuseum.org/research/pbs/pngherps/index.html). The *vertical line* is the international border between Papua New Guinea to the east and the Indonesian province of Papua to the west. Samples of *M. boeleni* from Papua Indonesia came from the *dashed box* region (*1*) and the single *M. boeleni* from Papua New Guinea came from the *dashed circle* region (*2*). Although precise localities are known, we draw vague localities for protection of this species (Stuart et al. 2006). Samples of *M. amethistina* came from locality (*3*) and *M. viridis* came from localities (*3*) and (*4*)

Guinea are CITES signatories and regulate trade of *M. boeleni* as a CITES Appendix II listed species. Virtually nothing is known about the natural history, ecology, or physiology of this montane endemic python. In addition, there is no data on population size, structure, or genetics of this species despite its conservation importance.

The current distribution of montane forest in New Guinea is a large swath of habitat along the New Guinea central cordillera as well as several isolated mountain ranges that are separated from the central cordillera by lowland forests. Very little precise distributional data is available for *M. boeleni* but it is found throughout the central cordillera as well as in allopatric populations on the Huon Peninsula and Goodenough Island (Fig. 1). It is unknown if *M. boeleni* in the central cordillera represent one contiguous interbreeding population or a series of isolated mountain-top populations with limited, reduced, or no gene flow with one another, but limited locality data suggests the latter as there appear to be two disjunct populations in the central cordillera (Fig. 1). The two known allopatric populations on the Huon Peninsula and Goodenough Island are likely currently genetically isolated from the central cordillera population(s) because the intervening lowland forest is not suitable habitat. *Morelia boeleni* therefore consists of multiple allopatrically distributed populations that may be lineages of a single or multiple species.

Historically, live specimens *M. boeleni* have been imported into the USA since the mid 1970s. International Species Information System (ISIS) records indicate that 127 legal specimens have entered zoological institutions and currently 44 (21 male; 23 female) of those specimens are alive. There are 102 (38 male; 64 female) specimens accountable in private ownership; this population includes animals in USA, UK, Spain, Japan, and Germany. Most of these specimens are thought to have originated from one population found in the Western Highlands of Papua, Indonesia. This species has gained the reputation of being difficult to maintain and reproduce in captivity. However, this reputation has diminished due to current natural history studies (Spataro and Baldogo: http://www.boelenspythons.com/home.html) and exporters offering captive hatched specimens. Throughout their short captive history, there has been a concentrated effort at breeding this species yet efforts have been successful only four times, with none of these occurring in zoological institutions. Current wild populations seem to be non-threatened, receiving little pressure from its two known predators: humans and the New Guinea Harpy Eagle (*Harpyopsis novaeguineae*). Their status may change, however, as the human population increases or more

Fig. 2 Boelen's python (*M. boeleni*) from Central Highlands, Papua Indonesia (photo M. Spataro)

natural resources are discovered on the island resulting in more habitat destruction. For these reasons this species should be considered a target species in need of a well-structured wild and captive management plan.

Upon discovering this species, Brongersma (1953) described it as a member of the genus *Liasis* (Gray 1842), where it remained until Kluge (2003) placed it within *Morelia* (Gray 1842), based on morphological and genetic analysis. *Morelia boeleni* are referred to by the following common names: Boelen's Python, Black Python, and Sanca bulan. Genetic analysis has shown that this species' closest extant relative is *Morelia amethistina* (Harvey et al. 2000; Rawlings et al. 2008), which also occurs on the island of New Guinea.

The objectives of this study are to elucidate general patterns of genetic structure for *M. boeleni* and to understand the levels of genetic variation in captive animals in zoological parks that are used for captive breeding purposes. We use DNA sequence data from two mitochondrial (mtDNA) genes and one nuclear (nDNA) gene to examine the amount of genetic variation from wild and captive animals. The mtDNA cytochrome *b* (cytb) gene has been widely used to detect population and specific level variation in boas and pythons (Austin 2000; Keogh et al. 2001; Vences et al. 2001; Auliya et al. 2002; Rawlings and Donnellan 2003; Lawson et al. 2004; Rawlings et al. 2008; Tzika et al. 2008) as well as other snakes. The second mtDNA gene region sequenced, cytochrome oxidase I (COI), has also been used extensively in snakes, reptiles, and other animals (Daniels et al. 2002; Melville et al. 2004; Utiger and Schatti 2004; Plaisance et al. 2008). The nDNA gene region sequenced, melanocortin-1 receptor (MC1R), has been shown to be variable in snakes, lizards, and other vertebrates (Theron et al. 2001; Rosenblum et al. 2004, 2007; Fajardo et al. 2008). This is the first phylogeographic study for any montane New Guinea reptile. As such these genetic data will provide a better understanding of estimates of the timing of population divergence and inferences of the historical population-level processes that generate and sustain the current patterns of genetic variation. Results of this study have implications for conservation of this species in the wild as well as providing important genetic data for captive breeding programs.

Materials and methods

We examined 108 specimens representing three python species from New Guinea. Two congeners of *M. boeleni*, *M. amethistina* and *M. viridis*, were incorporated to compare molecular divergence and diversity within the genus. DNA was extracted from 90 *M. boeleni* shed skins or tissue, and from liver from six *M. amethistina*, and 12 *M. viridis*, using a guanidine thiocyanate or ammonium acetate salt extraction protocol (http://socrates.berkeley.edu/~fujita/protocols/DNA_Extraction.pdf). Sheds of *M. boeleni* were donated from various zoological parks and private collectors (see "Appendix"). One nuclear (MC1R) and two mitochondrial (cytb and COI) gene fragments were amplified via PCR for each individual; each reaction contained the following: 25–50 ng template, 5 pmoles each primer (see Table 1 for primer sequences), 1.25 nmoles each dNTP, 1× PCR Buffer (New England Biolabs, Ipswich, MA), 0.5 units Taq polymerase (New England Biolabs, Ipswich, MA), and nuclease-free H_2 0–25 µl. Primers for MC1R were designed from squamte sequences in GenBank. Amplicons were purified using Exo-SAP IT (USB Corp., Cleveland, OH). Cleaned products were cycle sequenced with Big Dye 3.1 (Applied Biosystems, Foster City, CA) following manufacturer's protocols. Excess dye terminator was removed via sephadex column filtration, and products were electrophoresed on an ABI-3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence output was aligned and edited with Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI).

Of the 90 individuals of *M. boeleni*, 17 total sheds were classified as 'wild-caught', 16 from Indonesian Papua, and

Table 1 Primers used in this study

Name	Gene	Use	Sequence 5′–3′	Position*	Reference
M526	cytb	Amp./Seq.	ACCTGCGGCCTGAAAAAC	14925–14942	This study
M527	cytb	Amp.	GCTTTGGTTTACAAGAACAATGCT	16124–16101	This study
H15149	cytb	Seq.	AAACTGCAGCCCCTCAGAATGATATT	15379–15355	Kocher et al. 1989
VF1 5	COI	Amp./Seq.	TTCTCAACCAACCACAAAGACATTGG	6195–6220	Ivanova et al. 2006
VR1 5	COI	Amp./Seq.	TAGACTTCTGGGTGGCCAAAGAATCA	6901–6879	Ivanova et al. 2006
G482	MC1R	Amp./Seq.	TCAGCAACGTGGTGGA		This study
G480	MC1R	Amp./Seq.	ATGAGGTAGAGGCTGAAGTA		This study

* Position in *Python regius* mitochondrial genome

one museum specimen (BPBM 11611) was collected ~1200 km away in Morobe Province in eastern Papua New Guinea (PNG) (Fig. 1). The remaining 73 individual *M. boeleni* are of unknown origin but are presumed to have come from Indonesia as this has been the source of all known exports. All *M. amethistina* were from one locality (Milne Bay Province, PNG) and the *M. viridis* were collected from two localities (Sandaun and Milne Bay Provinces, PNG) ~1600 km distant (Fig. 1; "Appendix").

To compliment the empirical data, we performed a power analysis in order to assess the ability to detect presence of rare haplotypes with a sample of this size (16). Under the conservative assumption that these individuals were collected from the same locality, we calculated the probability of detecting more than one haplotype ($P_{>1}$) as $1 - \rho^{16}$, while varying the proportion (ρ) of the most common haplotype in the population between 0.5 and 1.

Results

All wild-caught specimens from Indonesia and all captive individuals of *M. boeleni* were genetically uniform across all three loci. No single nucleotide polymorphisms or insertion/deletion mutations were detected from the 1,418 bp of total aligned sequence data (cytb: 370 bp; COI: 553 bp; MC1R: 495 bp). For the nuclear locus no heterozygotes were detected as inferred from lack of double peaks in the electropherograms (Brumfield et al. 2003). The single haplotype seen in the cytb fragment sequenced from our Indonesian samples was identical to that obtained by Harvey et al. (2000). The single individual from eastern New Guinea (Morobe Province, PNG) ~1200 km distant from the Indonesian locality differed from the western samples by ≤1.1% sequence divergence for both mitochondrial genes, but the MC1R sequence was identical to the Indonesian *M. boeleni*. In contrast, sequences from the six *M. amethistina* and 12 *M. viridis* both yielded multiple haplotypes for each gene with mean intrapopulation divergence ranging from 0.24 to 0.41% and mean interpopulation divergence ranging from 6.8 to 7.5% for the mitochondrial genes and mean intrapopulation divergence ranging from 0.20 to 0.38% for the nuclear locus (Table 2). Results from the power analysis are shown in Fig. 3 and all sequences are deposited in GenBank (FJ864817-FJ865133).

Discussion

The lack of genetic variation for all 89 *M. boeleni* from wild-caught and captive sheds from Indonesia was

Table 2 Number of haplotypes and mean inter and intrapopulation values for the three loci sequenced for three species of *Morelia*

Gene		*Morelia* species (*N*)		
		M. amethistina (6)	*M. boeleni* (90)	*M. viridis* (12)
COI	Haplotypes	2	2[1 + 1]	4[3 + 1]
	Mean interpopulation divergence	N/A	0.0111	0.0688
	Mean intrapopulation divergence	0.0036	0	0.0024,0
cytb	Haplotypes	3	2[1 + 1]	5[3 + 2]
	Mean interpopulation divergence	N/A	0.0082	0.0757
	Mean intrapopulation divergence	0.0027	0	0.0041, 0.0027
MC1R	Haplotypes	2	1	2
	Mean interpopulation divergence	N/A	0	0
	Mean intrapopulation divergence	0.0038	0	0.0020, 0

Numbers in brackets denote numbers of haplotypes in each population

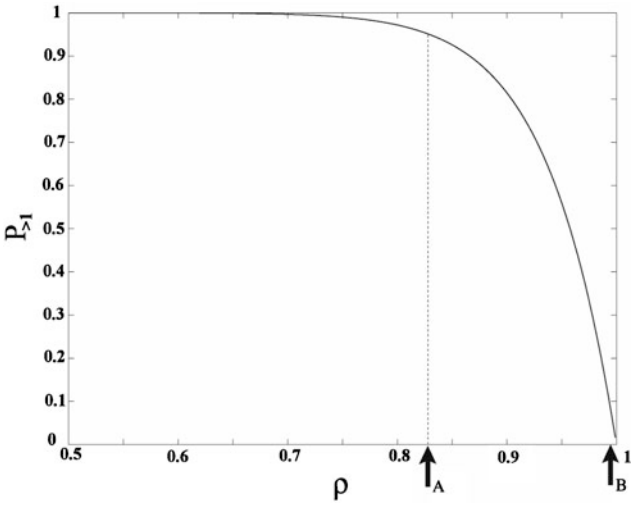

Fig. 3 A *power curve* representing the probability of detecting more than one haplotype ($P_{>1}$) in a sample of 16, given the known proportion of the most common haplotype (ρ). The probability to the right of A ($\rho = 0.83$) is less than 0.95, and in a population where the $\rho > 0.996$ (B), a sample of this size will fail to detect diversity in an average of 95% of cases

unexpected. Although only 16 of the 89 Indonesian *M. boeleni* were recorded as wild-caught we would still expect to detect some degree of haplotype diversity with this sample size, given the results from the congeners and molecular results from other pythons (Table 2; Austin 2000; Harvey et al. 2000; Rawlings and Donnellan 2003; Rawlings et al. 2008). These results may be attributed to differences in lineage histories, however, results of the power analysis suggest that a sample of this size is likely to reveal diversity even when a single haplotype comprises the majority of a population. While an analysis that incorporated the 2 N nuclear data would have more power to detect diversity (>95% detectable on average when a single allele is 91% of the population and <5% when a single allele is 99.8% of the population). The single *M. boeleni* from eastern New Guinea (Morobe Province, PNG) showed limited mtDNA divergence [COI (0.82%); cytb (1.1%)] despite being 1200 km distant. In contrast, *M. viridis* was sampled from two localities 1600 km apart and showed an almost ten-fold increase in mtDNA divergence and increased haplotype diversity [COI (6.8%), 4 haplotypes; cytb (7.5%), 5 haplotypes]. For the nuclear locus MC1R, *M. amethistina* from a single population showed intrapopulation level divergence (0.88%) for two haplotypes and *M. viridis* from two populations also showed intrapopulation level divergence (0.20%). These data from the two congeners closely mirror data collected for other pythons and suggests that *M. boeleni* shows reduced genetic diversity and divergence at both mtDNA and nuclear genomes (Austin 2000; Harvey et al. 2000; Rawlings and Donnellan 2003; Rawlings et al. 2008).

Low levels of genetic variability have been observed in many other vertebrates (O'Brian et al. 1985; Gray 1995; Kretzmann et al. 1996; Rivera et al. 2006; Vargas-Ramirez et al. 2007). Reduced levels of genetic variability are typically explained by historical bottleneck(s) in population size from either natural or human mediated processes. Inbreeding depression results from an increase in homozygosity and this loss of genetic variability has a variety of negative consequences including decreased fitness from reduced fecundity and lower survival. In addition, small or declining populations may be susceptible to mutational meltdown where deleterious mutations do not have the opportunity to be eliminated by natural selection (Amos and Balmford 2001).

The genetic uniformity of all 89 *M. boeleni* from Indonesia can best be explained by all our samples from captive and wild-caught animals coming from a single highly genetically uniform population. We only have precise locality data for four sheds collected from the wild by MS. The other sheds from wild-caught animals and the one shed from the USF&W confiscation may or may not come from our one known Indonesian locality but this would be the most parsimonious assumption based on the genetic data. Even if this were the case, the lack of haplotype diversity is surprising as most wild reptile populations have moderate amounts of haplotype diversity that would be detected with this sample size and is evident from our much smaller congener samples (Austin 2000; Harvey et al. 2000; Rawlings and Donnellan 2003; Rawlings et al. 2008). Given the broad, allopatric, and topographically varied distribution of *M. boeleni* our a priori expectation was that the species would show at least some degree of genetic variation even if most samples were from a single locality. One possible explanation for the genetic uniformity of a single population of *M. boeleni* would be a recent bottleneck associated with paleoclimatological oscillations. Montane reptiles may be particularly sensitive to rapid climate change and alterations or fluctuations in environmental conditions that shift populations up or down an elevational gradient may lead to genetic bottlenecks as one or a few individuals are able to colonize new climate suitable elevations. In particular, multiple rapid oscillations of climate may cause severe bottlenecks. Our results are still surprising given that examination of genetic diversity of populations that are resulted from postglacial recolonization still show moderate haplotype diversity (Clark et al. 2003; Fuerst and Austin 2004; Ursenbacher et al. 2006).

Future research will be needed to obtain genetic material from *M. boeleni* from across its range throughout the central cordillera and in particular the two known allopatric populations on the Huon Peninsula and Goodenough Island (Fig. 1). In addition, use of more variable markers such as microsatellites may provide further refinement of our

estimates of genetic structure in *M. boeleni* (Jordan et al. 2002). Further natural history studies are needed to increase our knowledge on this species. This data will hopefully help unlock the secrets as to why this is such a difficult species to reproduce in captivity and in return larger captive populations will be established. Institutions maintaining this species are encouraged to put forth more effort to assure their success with this species based on genetic and natural history information. Current CITES status should remain in effect, with better regulations of exportation on specimens originating from Indonesia.

Genealogical relationships and the harbored genetic diversity are important data for the use of developing a comprehensive captive breeding plan for any threatened or endangered species (Miller 1995). This study highlights the important need for further genetic sampling of *M. boeleni* from precise localities from across its range in order to assess if this species is actually highly genetically uniform and thus susceptible to inbreeding depression and in need of increased conservation protection and management.

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Appendix

See Table 3

Table 3 Material examined

Specimen ID	Species	Sex	Status
MB1	*boeleni*	F	Wild-caught[a]
MB2	*boeleni*	M	Wild-caught[a]
MB3	*boeleni*	F	Wild-caught[a]
MB4	*boeleni*	F	Wild-caught[a]
MB5	*boeleni*	F	Unknown
MB6	*boeleni*	F	Unknown
MB7	*boeleni*	M	Unknown
MB8	*boeleni*	M	Unknown
MB9	*boeleni*	M	Unknown
MB10	*boeleni*	M	Unknown
MB11	*boeleni*	F	Unknown
MB12	*boeleni*	F	Unknown
MB13	*boeleni*	F	Captive-bred
MB14	*boeleni*	M	Wild-caught[a]
MB15	*boeleni*	F	Unknown
MB16	*boeleni*	M	Unknown
MB17	*boeleni*	F	Unknown
MB18	*boeleni*	F	Unknown
MB19	*boeleni*	M	Unknown
MB20	*boeleni*	F	Unknown
MB21	*boeleni*	M	Unknown
MB22	*boeleni*	F	Unknown
MB23	*boeleni*	F	Unknown
MB24	*boeleni*	M	Unknown
MB25	*boeleni*	M	Unknown
MB26	*boeleni*	F	Unknown
MB27	*boeleni*	M	Unknown
MB28	*boeleni*	F	Unknown
MB29	*boeleni*	F	Wild-caught[a]
MB30	*boeleni*	M	Wild-caught[a]
MB31	*boeleni*	F	Wild-caught[a]
MB32	*boeleni*	F	Unknown
MB33	*boeleni*	F	Unknown
MB34	*boeleni*	M	Unknown
MB35	*boeleni*	M	Wild-caught[a]
MB36	*boeleni*	F	Captive-bred
MB37	*boeleni*	F	Captive-bred
MB38	*boeleni*	F	Captive-bred
MB39	*boeleni*	M	Captive-bred
MB40	*boeleni*	M	Captive-bred
MB41	*boeleni*	F	Unknown
MB42	*boeleni*	F	Unknown
MB43	*boeleni*	M	Unknown
MB44	*boeleni*	M	Unknown
MB45	*boeleni*	–	Unknown
MB46	*boeleni*	–	Unknown
MB47	*boeleni*	–	Unknown
MB48	*boeleni*	–	Unknown
MB49	*boeleni*	–	Unknown
MB50	*boeleni*	–	Unknown
MB51	*boeleni*	–	Unknown
MB52	*boeleni*	–	Unknown
MB53	*boeleni*	–	Unknown
MB54	*boeleni*	–	Unknown
MB55	*boeleni*	–	Unknown
MB56	*boeleni*	–	Unknown
MB57	*boeleni*	–	Unknown
MB58	*boeleni*	–	Unknown

Table 3 continued

Specimen ID	Species	Sex	Status
MB59	*boeleni*	–	Unknown
MB60	*boeleni*	M	Unknown
MB61	*boeleni*	F	Unknown
MB62	*boeleni*	M	Wild-caught[a]
MB63	*boeleni*	F	Wild-caught[a]
MB64	*boeleni*	M	Unknown
MB65	*boeleni*	F	Unknown
MB66	*boeleni*	F	Unknown
MB67	*boeleni*	M	Captive-bred
MB68	*boeleni*	F	Captive-bred
MB69	*boeleni*	F	Captive-bred
MB70	*boeleni*	F	Captive-bred
MB71	*boeleni*	F	Unknown
MB72	*boeleni*	M	Unknown
MB73	*boeleni*	–	Unknown
MB74	*boeleni*	F	Unknown
MB75	*boeleni*	F	Unknown
MB76	*boeleni*	F	Unknown
MB77	*boeleni*	–	Unknown
MB78	*boeleni*	–	Unknown
MB79	*boeleni*	–	Unknown
MB80	*boeleni*	–	Unknown
MB81	*boeleni*	–	Unknown
MB82	*boeleni*	–	Unknown
MB83	*boeleni*	M	Wild-caught[a]
MB84	*boeleni*	M	Wild-caught[a]
MB85	*boeleni*	F	Wild-caught[a]
MB86	*boeleni*	F	Wild-caught[a]
MB87	*boeleni*	M	Unknown[b]
MB88	*boeleni*	–	Captive-bred
MB89	*boeleni*	–	Wild-caught[a]
BPBM 11611	*boeleni*	–	Wild-caught[c]
CCA4515	*amethistina*	–	Wild-caught[d]
CCA5050	*amethistina*	–	Wild-caught[d]
CCA5051	*amethistina*	–	Wild-caught[d]
CCA5153	*amethistina*	–	Wild-caught[d]
CCA5192	*amethistina*	–	Wild-caught[d]
CCA5296	*amethistina*	–	Wild-caught[d]
CCA3538	*viridis*	–	Wild-caught[e]
CCA3580	*viridis*	–	Wild-caught[e]
CCA4598	*viridis*	–	Wild-caught[d]
CCA4599	*viridis*	–	Wild-caught[d]
CCA4677	*viridis*	–	Wild-caught[d]
CCA4678	*viridis*	–	Wild-caught[d]
CCA4679	*viridis*	–	Wild-caught[d]
CCA4680	*viridis*	–	Wild-caught[d]
CCA4978	*viridis*	–	Wild-caught[d]
CCA5072	*viridis*	–	Wild-caught[d]

Table 3 continued

Specimen ID	Species	Sex	Status
CCA5195	*viridis*	–	Wild-caught[d]
CCA5196	*viridis*	–	Wild-caught[d]

[a] Papua Province, Indonesia

[b] USFWS confiscation

[c] Morobe Province, Papua New Guinea

[d] Milne Bay Province, Papua New Guinea

[e] Sandaun Province, Papua New Guinea

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