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A comparison of DNA barcoding markers in West African frogs

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Abstract.—DNA barcoding has been proposed as a means of quick species identification using a short standardised segment of DNA. The established barcode gene for animals—the mitochondrial gene cytochrome oxidase one (CO1)—has been plagued by primer failure and low species identification success in amphibians. We investigate the accuracy of CO1 barcoding with a new dataset of West African frogs using the universal CO1 primers and new amphibian-specific CO1 primers in comparison to a proposed alternative DNA barcode for amphibians—the mitochondrial ribosomal 16s gene (16s). Research was performed using 134 specimens, comprising 21 species collected in Ghana, a global biodiversity hotspot with a deficiency of amphibian barcoding resources. These species represent 55% of amphibian species (58% of amphibian families) that are known in the area from surveys from 1988 to 2009. We found nearly a 50% increase in PCR amplification success using the amphibian-specific CO1 primers compared to the universal CO1 primers. However, the overall amplification and sequencing success of the amphibian-specific CO1 primers was low (78%) compared to the 16s gene (100%). Neither marker has a clear advantage in terms of barcoding gap; comparisons of intraspecific and interspecific variation for these markers were similar for the species we examined. Considering the qualities a barcoding gene should possess, 16s outperformed CO1 in terms of ease of obtaining sequences, and given that 16s sequences are better represented for African frogs on GenBank, this marker had higher success in BLAST searches. With amphibian species in fast decline, more consideration should be given to the appropriateness of collecting CO1 barcodes for amphibians, especially as an extensive genetic database for 16s already exists that can accurately identify amphibians.

Key words.—DNA barcode; amphibians; cytochrome oxidase one (CO1); 16s rRNA; Ghana.

Introduction

Amphibians are an extremely endangered group of animals, with 32–41% categorised as threatened by the International Union for Conservation of Nature (IUCN 2015; Stuart *et al.* 2004). This is likely an underestimate as the number of described amphibian species is still increasing and there is insufficient population assessment and monitoring data to determine IUCN status on at least a third of the known amphibian species (Bickford *et al.* 2007;

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Fouquet *et al.* 2007; IUCN 2015; Köhler *et al.* 2005). Amphibians have morphologically different life stages, high phenotypic plasticity and high levels of cryptic diversity between species. These traits often make it difficult to correctly identify species, especially at early life-stages. DNA barcoding could assist scientists as a quick means of obtaining accurate species identifications. Collecting accurate data on a region's biodiversity through population census and species richness is crucial to determine vulnerability status and conservation priority for amphibian species (Bickford *et al.* 2007; Fouquet *et al.* 2007; Hebert *et al.* 2004; Maya-Soriano *et al.* 2012; Taylor & Harris 2012; Vences *et al.* 2005a, b; 2012; Waugh 2007).

In 2003, a barcoding system was established as a solution for rapid and accurate species identification (Hebert *et al.* 2003). In 2004, the Barcode of Life Database (BoLD) began compiling DNA barcodes for animal species using the only gene authorised for the animal kingdom in BoLD—a 648 bp region of the mitochondrial gene, cytochrome oxidase one (CO1). The CO1 gene has a high success rate at species identifications in numerous animal taxa including birds, fish, and many invertebrates (Hebert *et al.* 2003; Janzen *et al.* 2009; Pfenninger *et al.* 2007). Additionally, CO1 has shown success in identifying species with cryptic life stages, sexual dimorphism, and/or high phenotypic plasticity (Hebert *et al.* 2003; Hebert *et al.* 2004; Packer *et al.* 2009; Pfenninger *et al.* 2007; Alex Smith *et al.* 2013; Waugh 2007). However, as noted by many researchers, including DNA barcoding proponents, it is not realistic to expect one gene to be successful for accurate species identification across all animal taxa (DeSalle *et al.* 2005; Hebert *et al.* 2003; Janzen *et al.* 2009; Meier *et al.* 2006; Nielsen & Matz 2006; Taylor & Harris 2012; Vences *et al.* 2005b). Some issues that prevent the CO1 gene from being an effective barcode for all animal groups include difficulty with primer success resulting from primer site variability as well as inaccurate species identifications due to different evolutionary rates of the CO1 gene in various taxa (Davison *et al.* 2009; DeSalle *et al.* 2005; Hickerson *et al.* 2006; Meier *et al.* 2006; Meyer & Paulay 2005; Nielsen & Matz 2006; Taylor & Harris 2012; Vences *et al.* 2005a, b; 2012; Waugh 2007). Other problems also arise when using a single gene to accurately identify species that have high hybridisation and introgression rates, recent species divergence, or homoplasy, but this problem extends beyond just CO1 (Hebert *et al.* 2003; Meyer & Paulay 2005; Murphy *et al.* 2013; Smith *et al.* 2008; Taylor & Harris 2012; Vences *et al.* 2005 a, b; 2012; Waugh 2007).

Amphibians have been one of the most problematic groups in terms of CO1 barcoding. Polymerase chain reaction (PCR) and sequencing of CO1 in amphibians has a history of low success (Davison *et al.* 2009; DeSalle *et al.* 2005; Maya-Soriano *et al.* 2012; Meier *et al.* 2006; Meyer & Paulay 2005; Smith *et al.* 2008; Taylor & Harris 2012; Vences *et al.* 2005a, b; 2012; *Waugh 2007*). When sequences are obtained, there is minimal success at species identification due to overlapping levels of intraspecific and interspecific variation (Fouquet *et al.* 2007; Maya-Soriano *et al.* 2012; Smith *et al.* 2008; Taylor & Harris 2012; Vences *et al.* 2005a, b). Since the barcoding movement began in 2004, CO1 barcoding of amphibians has been inundated with difficulties, and concerns have been raised whether another gene might be more suitable (Che *et al.* 2012; Maya-Soriano *et al.* 2012; Vences *et al.* 2005a, b). The 16s gene, which is widely used in amphibian systematics and taxonomy, has been proposed as an alternative DNA barcode to augment CO1 for additional confirmation of identification (Maya-Soriano *et al.* 2012; Vences *et al.* 2005a, b; 2012). However, new amphibian-specific CO1 primers, which have produced high amplification, sequencing and identification success in Malagasy Mantellids and Asian amphibians have now raised the question of whether using 16s as a

complementary barcoding gene is necessary for amphibian barcoding (Che *et al.* 2012; Jeong *et al.* 2013; Murphy *et al.* 2013; Vences *et al.* 2012). This research investigates the success of the new amphibian-specific primers for CO1 on species that have never been tested before.

West Africa is a region with impressive amphibian biodiversity, yet minimal DNA barcoding efforts (BoLD 2015; Ratnasingham & Hebert 2007). The Guinean rainforests in sub-Saharan Africa have been identified as a biodiversity hotspot with increasing environmental threats (Myers *et al.* 2000; Penner *et al.* 2011). Current rates of forest fragmentation and habitat degradation in Ghana are heavily impacting amphibian populations with one third of amphibians already considered threatened (Adum *et al.* 2013; Ofori-Boateng *et al.* 2013). Having DNA barcode profiles linked with voucher specimens will inform and assist in species monitoring and management. This area also appears to be one of the few refuges left on the planet that lacks evidence of chytrid infection and warrants close monitoring (Penner *et al.* 2013).

In this study, we evaluate and compare the CO1 and 16s genes for use as effective barcodes for West African amphibians. We use the universal CO1 primers, the new amphibian-specific CO1 primers, and the universal 16s primers on frogs collected from a fragment of the Guinean forest located in the Atewa Hills in the Eastern Region of Ghana (Fig. 1). We investigate the utility of the CO1 and 16s gene as DNA barcodes by comparing the quantity and quality of successful sequences obtained for both genes and their ability to successfully identify species.

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Materials and Methods

Sample and Collection

We analysed 134 frog specimens from the Atewa Hills in Ghana (Table 1). The specimens were collected 26–28 May 2011. All of the specimens are archived at the Burke Museum of Natural History and Culture, University of Washington, USA. The specimens were identified in the field at the time of collection. Tissue samples were harvested and flash frozen in liquid nitrogen and subsequently stored at -80 °C. All specimens are available for loan from the Burke Museum of Natural History and Culture (accession number 2011-176).

DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted from 117 of the 134 specimens using approximately 25 mg of liver tissue with a standard salt extraction method (MacManes 2013). DNA from the other 17 specimens was extracted from skin swabs using a Qiagen kit as part of a previous study (Penner *et al.* 2013). All PCR reactions were performed using EmeraldAmp® MAX PCR Master mix or EmeraldAmp® GT PCR Master mix. Primers used for 16s amplification were: 16SA-L: 5′ – CGCCTGTTTATCAAAAACAT – 3′ and 16SB-H: 3′ – CCGGTCTGAACTCAGATCACGT – 5′. Thermal cycle parameters for 16s were: initial denaturation for 3 min at 94 °C, followed by 30 s at 94 °C, 30 s at 56 °C, and 1 min at 72 °C for 35 cycles succeeded by 3 min at 72 °C for final extension (Vences *et al.* 2005b) (Table 1). The CO1 648 bp partial fragments were amplified first with the universal CO1 primers: LCO1490: 5′ – GGTCAACAAATCATAAAGATATTGG – 3′ and

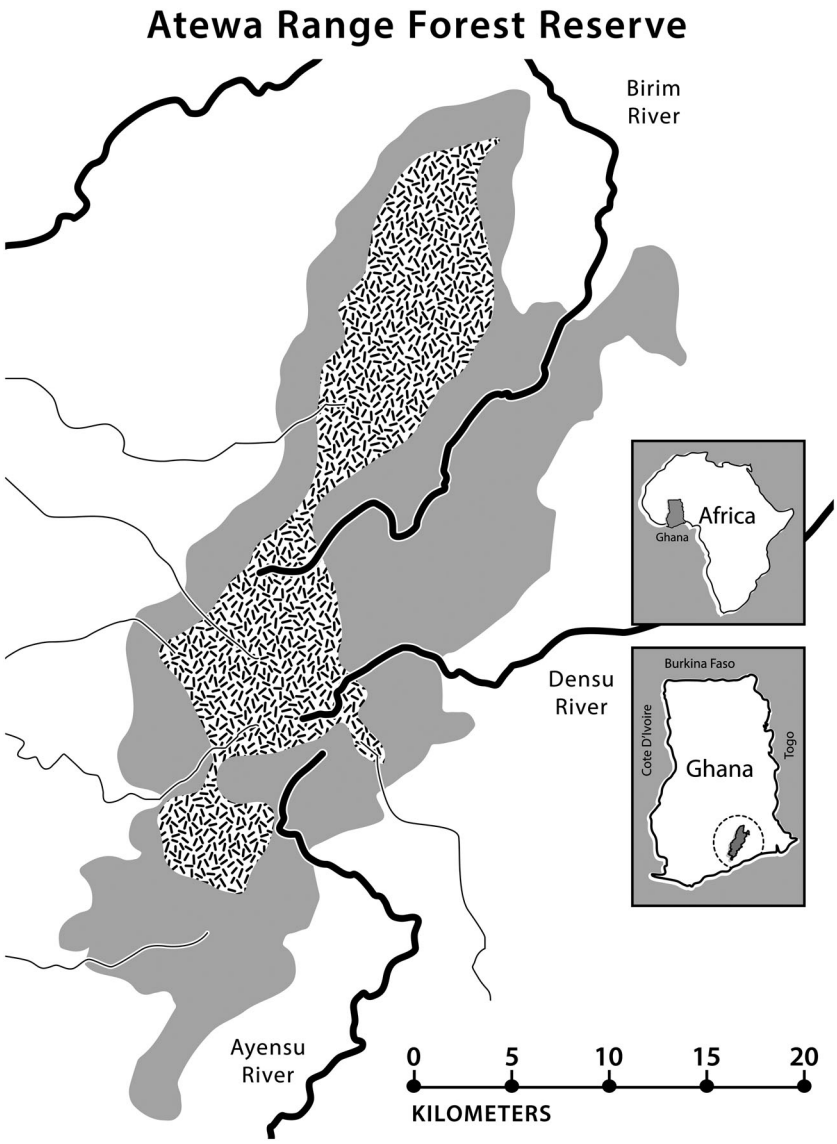

Figure 1. Map of the study area, Atewa Hill region, Ghana. The solid grey represents the Atewa Range and the interior spotted section is the Atewa Range Forest Reserve.

HCO2198: 5′ – TAAAACTTCAGGGTGACCAAAAAATCA – 3′ (Folmer *et al.* 1994). Thermal cycle parameters were: 5 min at 95 °C for initial denaturation, followed by 1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C for 35 cycles succeeded by 10 min at 72 °C for final extension (Che *et al.* 2012). Out of the 134 original samples, 77 samples were also amplified with new amphibian-specific CO1 primers: Chmf4: 5′ – TYTCWACWAAYCAYAAAGAYATCGG – 3′ AND Chmf4: 3′– ACTAAR-AARCCRGTRGGRCTYCA – 5′ (Che *et al.* 2012). Thermal cycle parameters for initial denaturation were set at 95 °C for 5 min, followed by 35 cycles for 1 min at 94 °C,

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Table 1. West African frog diversity included in the study. PCR was performed using the universal CO1 primers (CO1U), amphibian-specific CO1 primers (CO1A) and universal primers for 16s. Habitat preferences are F = Forest, S = Savanna and FB = Farmbush. IUCN listing key is LC – Least Concern, VU – Vulnerable, NT – Near Threatened, EN –Endangered and CR – Critically Endangered (Hillers *et al.* 2009; Hughes 1988; IUCN 2015; Kouamé *et al.* 2007; Leaché *et al.* 2006; Rödel & Agyei 2003; Rödel *et al.* 2005).

Family	Species	IUCN	Habitat	Sample size	16s	CO1U	CO1A
Arthroleptidae	*Arthroleptis poecilonotus*	LC	F, S, FB	13	13	13	10
	Astylosternus laticephalus	LC	F	2	2	2	2
Hyperoliidae	*Afrixalus dorsalis*	LC	F, S, FB	3	3	3	3
	A. nigiriensis	NT	F	6	6	6	5
	Hyperolius bobirensis	EN	F	22	22	22	9
	H. concolor cf.	LC	F, S, FB	1	1	1	1
	H. fusciventris	LC	F, S, FB	7	7	7	3
	H. picturatus	LC	F, S, FB	13	13	13	10
	H. sylvaticus	LC	F	13	13	13	3
	Kassina arboricola	VU	F	12	12	12	7
	Leptopelis occidentalis	NT	F	1	1	1	1
	L. spiritusnoctus	LC	F, FB	7	7	7	4
	Phlyctimantis boulengeri	LC	F, S, FB	6	6	6	4
Phryno-batrachidae	*Phrynobatrachus plicatus*	LC	F, FB	4	4	4	3
	P. species	-	F	2	2	2	1
	P. calcaratus	LC	F, S, FB	4	4	4	0
	P. tokba	LC	F	2	2	2	0
Pipidae	*Silurana tropicalis*	LC	F, S, FB	5	5	5	1
Ptychadenidae	*Ptychadena aequiplicata*	LC	F, FB	4	4	4	4
Pyxicephalidae	*Aubria subsigillata*	LC	F	6	6	6	5
Ranidae	*Amnirana albolabris*	LC	F, S, FB	1	1	1	1
Total attempted	21 species			134	134	134	77

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then 1 min at 46 °C, and then 1 min at 72 °C, followed by a final extension for 10 min at 72 °C. All PCRs were performed using BioRad© T100 Thermal Cyclers, and all products were visualised with gel electrophoresis. PCR products were sequenced in the forward and reverse directions for both CO1 and 16s (using all six primers listed above) through Genewiz, a sequencing servicing company, using an ABI 3730*xl* DNA Analyser. All sequences for CO1 and corresponding 16s sequences are submitted and available on BoLD under project code WAF. Additionally, 16s without corresponding CO1 sequences are available on GenBank (accession numbers KU166806-KU166858).

Data Analysis—Quality, Identification and Variation

Sequencing results were evaluated with Sequencher 5.0.1. After the primer sequences were removed, the remaining sequence was discarded and considered failed if any of the

following conditions were met: (1) there were fewer than 450 bp after forward and reverse alignment, (2) average sequence quality after forward and reverse alignment was below 75%, or (3) only one direction was sequenced and the quality was below 75%. When both directions were successfully sequenced, they were aligned and ambiguities were checked and individually adjusted if one direction was unambiguous. If an ambiguity was not able to be determined, it was left in the final submitted sequence. Since some of the CO1 sequences were obtained from either the universal primer or the amphibian-specific primer, they were aligned together prior to submission to the BoLD and GenBank databases. The average length and quality for CO1 and 16s sequences were calculated by averaging all successful sequences for each species (Table 2). We conducted BLAST searches by inputting the FASTA sequences in the nucleotide collection database (under option 'other') for each specimen using the Megablast search for highly similar sequences on GenBank for each 16s and CO1 sequence. Additionally, the CO1 sequences were put into the BoLD Identification Request tool to check identity. All 16s and CO1 sequences were aligned using MUSCLE v3.8.31 (Edgar 2004). We calculated GTR-corrected intraspecific and interspecific distances using DIVEIN web server (Deng *et al.* 2010).

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RESULTS

Quality and Identification

Of the 134 samples included in this study, 100% were successfully PCR amplified and sequenced for 16s with an average DNA quality of 95% (after trimming) and an average length of 565 bp. Values of overall sequence quality were calculated by the Sequencher 5.0.1 program. For the universal CO1 primer, 40 out of 134 samples were sequenced in both directions, with an average DNA quality of 97% and an average sequence length of 590 bp. Moreover, 15 specimens sequenced in the reverse direction only had 98% average quality and an average sequence length of 572 bp. The amphibian-specific primer for CO1 was attempted with 77 samples and 60 specimens were sequenced in both directions. These 60 samples had an average final length of 638 bp and average DNA quality of 96%. An additional 7 specimens were sequenced in only one direction, with an average length of 625 bp and an average DNA quality of 91%. Although, the subset for the amphibian-specific primers is smaller, the species included represent all families tested with the other two subsets. When CO1 sequences from both the universal primer and amphibian-specific primer were combined, there were a total of 80 successful sequences in both directions and 10 in one direction only.

Eight species failed for sequencing using the CO1 universal primers, but produced successful sequences using the amphibian-specific CO1 primer. Six of these species were in the Hyperoliidae family and the additional two were from the Phrynobatrachidae and Pipidae families. Two specimens, *Arthroleptis poecilonotus* (UWBM 05623) and *Hyperolius picturatus* (UWBM 05724) were successfully aligned using the reverse sequence from the universal primer and the forward sequence from the amphibian-specific primer.

Species Identification

Species identification success varied for 16s and CO1 using the BLAST and BoLD databases. Results are based on the highest identifiable taxonomic level. For example, if a

Table 2. BLAST and BoLD search results for CO1 and 16s identifications. No sequences for *Phrynobatrachus tokba* or *Leptopelis spiritusnoctus* were obtained for CO1. Percentages represent the amount of similarity in matched specimens on GenBank or BoLD databases. No Match represents species that did not match any species in the Anura order on BoLD.

Family	Species	16S	BLAST	CO1	BLAST	BoLD
Arthroleptidae	*Arthroleptis poecilonotus*	13	Species (97%)	8	Genus (93%)	Species (93%)
	Astylosternus laticephalus	2	Species (99%)	1	Genus (82%)	No Match
Hyperoliidae	*Afrixalus dorsalis*	3	Genus (98%)	2	Family (93%)	Species (90%)
	A. nigiriensis	6	Genus (89%)	5	Family (82%)	Genus (88%)
	Hyperolius bobirensis	22	Genus (94%)	7	Family (83%)	Genus (87%)
	H. concolor cf.	1	Species (99%)	1	Family (82%)	Genus (86%)
	H. fusciventris	7	Genus (89%)	6	Genus (85%)	No Match
	H. picturatus	13	Genus (99%)	10	Family (82%)	Genus (88%)
	H. sylvaticus	13	Genus (98%)	12	Family (84%)	Genus (88%)
	Kassina arboricola	12	Genus (97%)	7	Order (83%)	No Match
	Leptopelis occidentalis	1	Genus (98%)	1	Genus (89%)	Genus (96%)
	L. spiritusnoctus	7	Species (97%)	0	–	–
	Phlyctimantis boulengeri	6	Genus (97%)	4	Order (81%)	Genus (90%)
Phrynobatrachidae	*Phrynobatrachus plicatus*	4	Species (100%)	3	Order (81%)	Genus (84%)
	P. species	2	Genus (99%)	1	Order (79%)	No Match
	P. calcaratus	4	Species (99%)	2*	Order (82%)	Genus (84%)
	P. tokba	2	Species (100%)	0	–	–
Pipidae	*Silurana tropicalis*	5	Species (99%)	1	Species (99%)	Species (100%)
Ptychadenidae	*Ptychadena aequiplicata*	4	Species (96%)	4	Order (82%)	Genus (85%)
Pyxicephalidae	*Aubria subsigillata*	6	Species (98%)	6	Order (81%)	Genus (82%)
Ranidae	*Amnirana albolabris*	1	Species (91%)	1	Genus (83%)	Species (86%)
	21 species	134		82		

*Only two reverse sequences were obtained for *Phyrnobatrachus calcaratus*

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specimen is listed as being identified to the genus level, then it was not possible to identify the species. However, if a specimen was identified to the species level, then it is included as being correctly identified to higher taxonomic levels in the results listed below. BLAST results for 16s (out of 21 species) accurately identified 11 (52%) of the species to species level and all 21 (100%) to the genus level (Table 2). For CO1 (out of 19 species), only 1 (5%) species was identified to species level, 6 (32%) to genus, 12 (63%) to family (Table 2). The BoLD search was limited to CO1, as it is the only gene available for identification in the BoLD database. Of the 19 species we attempted to identify using the BoLD database, 4 (21%) were correctly identified at the species level, and 15 (79%) identified to genus. Four species provided no matches for the Anura Order in the BoLD database (Table 2).

Interspecific and Intraspecific Variation

Interspecific variation for both CO1 and 16s had similar ranges, however the CO1 range was about 5% higher than 16s. The lowest variation for 16s was 5% between *Hyperolius concolor* and *H. bobirensis,* and the maximum variation was 36% between *H. fusciventris* and *Phyrnobatrachus* sp. (Fig. 2). The interspecific variation range for CO1 had a low of 9% between *H. concolor cf.* and *H. bobirensis*, and a high at 43% between *H. sylvaticus* and *Phyrnobatrachus* sp. (Fig. 2). The 16s analysis showed an average interspecific variation of 28% between species, while CO1 had a slightly higher average in the range of 31–32% (Fig. 2). The levels of intraspecific variation between the 16s and CO1 showed high variability dependent on species, but none were above 2% for either gene (Fig. 3). However, there were different numbers of sequences used for comparisons between CO1 and 16s. While 16s had 134 sequences for comparison, there were only 82 sequences,

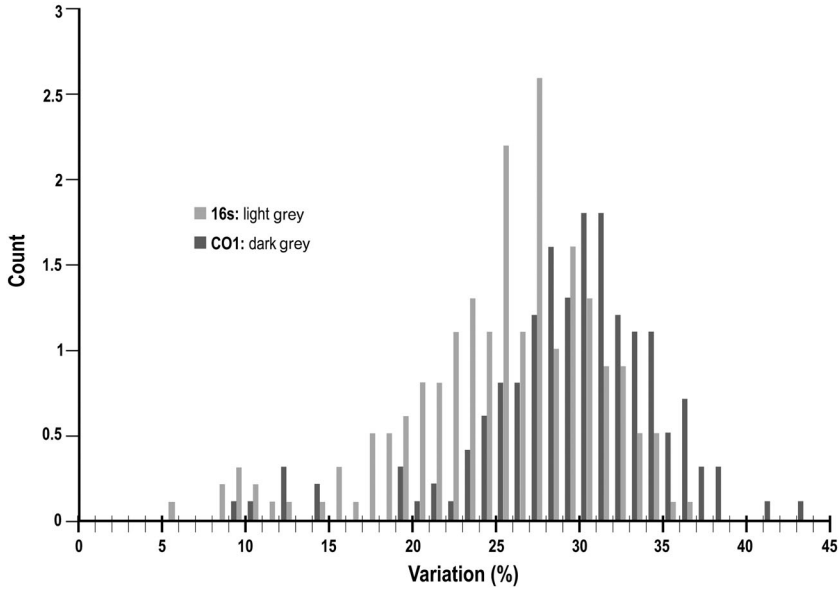

Figure 2. Interspecific variation for 16s (light grey) and CO1 (dark grey) of amphibians used in this study.

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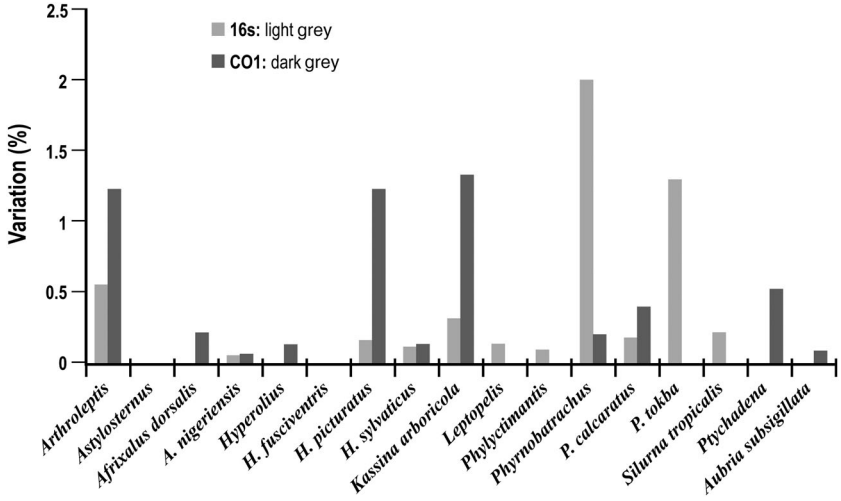

Figure 3. Intraspecific variation for 16s (light grey) and CO1 (dark grey) of amphibians used in this study. Species that had only one specimen sequenced were not incorporated in this analysis including: *Leptopelis occidentalis, Hyperolius concolor* cf. and *Amnirana albolabris* for both CO1 and 16s: additionally, two species were not calculated for CO1: *Astylosternus laticephalus* and *Silurana tropicalis*.

with 2 being reverse-only sequences (*Phrynobatrachus calcaratus*) for CO1. The number of specimens per species used also varied between the genes. There were 8 species from 16s and 6 species from CO1 results that had 5 or fewer representative specimens. Three species had only one specimen and could not be computed for either gene: *Hyperolius cf. concolor, Leptopelis occidentalis* and *Amnirana albolabris.* While an additional 3 species had only one specimen for CO1 and could not be computed: *Astylosternus laticephalus, Phrynobatrachus* sp. and *Silurana tropicalis*. There were also 2 species that had no successful sequences obtained for CO1 and could not be included: *Leptopelis spiritusnoctus* and *Phyrnobatrachus tokba*.

Discussion

In order to establish a robust DNA barcoding repository, there are standardisation criteria for all submissions to the BoLD database. Barcodes must be at least 500 bp, contain no more than 1% ambiguous bases, and both the forward and reverse directions are required (BoLD 2015; Ratnasingham & Hebert 2007). With respect to the species of West African amphibians studied, all of these criteria were met with the 16s gene. This was not the case for the CO1 gene, since the specimens with only one direction sequenced do not qualify as viable barcodes. As for specimens that had both directions sequenced for CO1 and 16s, very few ambiguities were present for either gene after trimming. PCR for 16s was straightforward in that one standard protocol was used with 100% success. Initial attempts for both the universal and amphibian-specific primers for CO1 failed. Given the history of difficulties known with using the CO1 universal primers on amphibians, the low success rate (30%) of the universal primers is not surprising. Overall, the success of the amphibian-specific primers (78%) had an increase of 46% over the universal primers. It is probable

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that the success of CO1 with the amphibian-specific primers would increase if a separate temperature gradient experiment was performed for each species or family. However, multiple temperature gradients were not done as performing multiple PCR reactions greatly increases cost, time and resources used. Despite a definitive improvement of PCR product with the amphibian-specific CO1 primers, the question remains whether it is a good enough improvement to validate the continued use of CO1 as the amphibian barcode compared to 16s.

The objective of creating a DNA barcoding database is to build a reference library that can be used to identify unknown specimens. Specimen sequences were run through the BoLD and GenBank's identification tools to determine their suitability for amphibian identification (Table 2). The CO1 database, BoLD, successfully identified 21% of specimens to the species level. This was an improvement in species level identifications compared to CO1 results in GenBank by 16%. In comparison to GenBank, there was also a 47% improvement to genus level identification using BoLD. Results for identifications through GenBank's BLAST tool favoured 16s with 52% of the specimens identified to the species level, while only 5% of the specimens were identified to species level for CO1. The bias towards 16s as a more accurate marker lies with the problematic nature of obtaining CO1 sequencing for amphibians, which has created a vast discrepancy in the number of 16s and CO1 sequences available on GenBank. Currently, there is an extensive library of 16s sequences for amphibians available on GenBank. According to a search for available amphibian sequences on GenBank, there are 1 851 CO1 sequences, while there are 37 637 available sequences for 16s (GenBank 2015). There are too few representative amphibian species for CO1 available on GenBank for accurate species identifications. Amphibian CO1 sequences available in BoLD number 24 124, representing 2 672 species (BoLD 2015; Ratnasingham & Hebert 2007). While this number seems impressive given the recent advent of the barcoding movement, a total of 1 171 (38%) of these species have fewer than 3 representative reference sequences with at least 500 bp. When attempting to identify specimens in this study, BoLD consistently had no results found unless the sequence was searched with the 'All Records' search option. This search option had better results, but most barcode matches were unpublished and inaccessible (BoLD 2015; Ratnasingham & Hebert 2007). One solution to this problem is to re-sequence CO1 for all amphibian species that have existing 16s sequences available. However, the 16s gene has a robust phylogenetic signal and can be used to accurately identify species despite an incomplete reference database (Vences *et al.* 2012). Perhaps, instead of developing a complete database of amphibian CO1 sequences, a more effective solution would be to continue sequencing 16s for amphibians, especially considering amphibians are facing a present-day extinction crisis.

Interspecific variation for both CO1 and 16s showed similar patterns as previous studies with 5% threshold for 16s and 9% for CO1 (Fouquet *et al.* 2007; Vences *et al.* 2005a, b; 2012; *Xia et al. 2012*). Both genes had similar intraspecific variation trends within the 0–2% range. In the context of determining predictive species thresholds, there are too few specimens per species and too few species for this study to provide any conclusive evidence to support CO1 or 16s as a superior gene for species identification in West African amphibians.

According to Hebert *et al.* (2003), for the original proposal of a CO1 barcoding system, a barcode gene should have reliable universal primers, cost-efficient methods, and an efficient standard protocol for PCR and sequencing. Despite advances of a more efficient primer for CO1, there is still no standard method for obtaining consistent CO1

sequences or identification results for amphibians. Even degenerate amphibian-specific primers needed constant adjustments to the PCR protocol to obtain results that yielded less than 80% success. Given increasing threats on amphibian species, the time available to establish an extensive CO1 amphibian reference library is limited. Without a complete reference database, CO1 will not be able to accurately identify unknown specimens and will always need a backup gene to confirm species identity. Having to sequence two genes increases time, resources and costs, which is contrary to the concept of quick species identifications using a barcoding system. It is obvious the information from a CO1 database would be of great value to further the research of amphibian evolutionary relationships. However, evidence from this study does not support that it is an ideal barcoding gene for West African amphibians. In comparison, the existing reference database, ease of PCR and sequencing, and historical high identification success rates of 16s align with the definition of a more appropriate barcoding gene for amphibians.

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