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NEWS AND VIEWS

OPINION

Cold Code: the global initiative to DNA barcode amphibians and nonavian reptiles

ROBERT W. MURPHY,*† ANDREW J. CRAWFORD,‡§ AARON M. BAUER,¶ JING CHE,* STEPHEN C. DONNELLAN,** UWE FRITZ,†† CÉLIO F.B. HADDAD,‡‡ ZOLTÁN T. NAGY,§§ NIKOLAY A. POYARKOV,¶¶ MIGUEL VENCES,*** WEN-ZHI WANG* and YA-PING ZHANG*†††

*State Key Laboratory of Genetic Resources and Evolution State, and Yunnan Laboratory of Molecular Biology of Domestic Animals, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China, †Centre for Biodiversity and Conservation Biology, Royal Ontario Museum, 100 Queen's Park, Toronto, Canada, M5S 2C6 ‡Department of Biological Sciences, Universidad de los Andes, A.A. 4976, Bogotá, Colombia, §Smithsonian Tropical Research Institute, Apartado 0843-03092, Panamá, Republic of Panama, ¶Department of Biology, Villanova University, 800 Lancaster Avenue, Villanova, PA 19085-1699, USA, **Evolutionary Biology Unit, South Australian Museum, North Terrace, Adelaide SA 5000, Australia, ††Museum of Zoology, Koenigsbruecker Landstr. 159, Dresden 01109, Germany, ‡‡Departmento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, Av. 24 A 1515, Rio Claro, 13506-900, São Paulo Brazil, §§Joint Experimental Molecular Unit, Royal Belgian Institute of Natural Sciences, Brussels, Belgium, ¶¶Department of Vertebrate Zoology, Moscow MV Lomonosov State University, Moscow 119991, Russia, ***Zoological Institute, Technical University of Braunschweig, Mendelssohnstr. 4, Braunschweig, 38106, Germany, †††Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Kunming 650091, China

Abstract

DNA barcoding facilitates the identification of species and the estimation of biodiversity by using nucleotide sequences, usually from the mitochondrial genome. Most studies accomplish this task by using the gene encoding cytochrome oxidase subunit I (*COI*; Entrez *COX1*). Within this barcoding framework, many taxonomic initiatives exist, such as those specializing in fishes, birds, mammals, and fungi. Other efforts center on regions, such as the Arctic, or on other topics, such as health. DNA barcoding initiatives exist for all groups of vertebrates except for amphibians and nonavian reptiles. We announce the formation of Cold Code, the international initiative to DNA barcode all species of these 'cold-blooded' vertebrates. The project has a Steering Committee, Coordinators, and a home page. To facilitate Cold Code, the Kunming Institute of Zoology, Chinese Academy of Sciences will sequence *COI* for the first 10 specimens of a species at no cost to the steward of the tissues.

Keywords: amphibia, barcoding, COI, crocodylia, identification, lepidosauria, testudines

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Since its origin (Hebert *et al.* 2003), DNA barcoding has proven to be an invaluable source of information, not only in its applications to forensics (Baker & Palumbi 1994; Begerow *et al.* 2010) and conservation, including the discovery of undescribed species (Neigel *et al.* 2007; Vernooy *et al.* 2010; Crawford *et al.* 2013) but also for documenting environmental change (Crawford *et al.* 2010), identifying taxa in need of further systematic study (Vieites *et al.* 2009; Francis *et al.* 2010), verifying museum tissue collections (Puillandre *et al.* 2012), identifying invasive species (Armstrong & Ball 2005; Crawford *et al.* 2011), tracking progress in the Genome 10K initiative (Haussler *et al.* 2009; Wong *et al.* 2012) and many other applications involving the identification of organisms. Its functionality lies in the sequencing of one standardized genetic marker, in the case of animals cytochrome oxidase subunit I (*COI*; Entrez Gene *COX1*, plus synonyms *COXI* and *MT-CO1*), from many individuals and taxa, and populating an online database of reference sequences from vouchered specimens that represent the diversity of the study group (Ratnasingham & Hebert 2007). Curated DNA barcodes can act as a digital identifier and as a link between the genetic data available in

Correspondence: Robert W. Murphy, Fax: 1-416-586-5553; E-mail: bob.murphy@utoronto.ca

biological material and the taxonomic expertise represented by the curated specimen. Although barcode sequences can, in some cases, also serve to explore phylogenetic and phylogeographical questions, their primary utility lies in species identification and discovery.

Several global barcoding initiatives are underway. The barcode of life project for fishes (http://www.fishbol.org/) has made great headway in obtaining DNA barcodes by sequencing for almost 80 000 specimens representing almost 9 000 species (i.e. about one-third of the species) and more than 2 000 unnamed clusters of species. Some important economic applications involve the identification of marketplace fishes (Steinke *et al.* 2009) and seafood (Handy *et al.* 2011). The All Bird Barcoding Initiative (ABBI) has processed more than 26 000 specimens and over 4 000 species to date (http://www.barcodingbirds.org/) (Kerr *et al.* 2009). Similar consortia using self-explanatory acronyms include Bee-BOL, HealthBOL, MarBOL (marine life), PolarBOL, SharkBOL, SpongeBOL and TrichopteraBOL. Additional initiatives are focused on mosquitoes, fungi, coral reefs, lepidopterans, mammals, protists, tephritids and plant pathogens (http://www.barcodeoflife.org/content/community/projects). However, no coordinated efforts so far involve either amphibians or nonavian reptiles (i.e. crocodiles, squamates, tuataras, turtles and hereafter termed reptiles).

Amphibians and reptiles are important economically as a source of food, in the pet trade, in traditional Asian medicine, and as environmental indicator species for global climate change (e.g. van Dijk *et al.* 2000; Carpenter *et al.* 2005; Mohneke *et al.* 2009; Sinervo *et al.* 2010; Nijman & Shepherd 2011). Many species are now in danger of becoming extinct due to the combined action of habitat destruction, emerging pathogens, chemical pollutants and climate change. Collectively, they are the most imperilled group of vertebrates (Hoffmann *et al.* 2010). Molecular genetical approaches have identified many cryptic species (e.g. Fouquet *et al.* 2007; Oliver *et al.* 2009; Vieites *et al.* 2009; Vargas-Ramírez *et al.* 2010; Funk *et al.* 2011; Jansen *et al.* 2011; Kindler *et al.* 2012), and the rate of new species descriptions per year shows no sign of slowing (Glaw & Köhler 1998; Köhler *et al.* 2005; Uetz 2010). Unfortunately, in many cases we appear to be losing species before they are described and before we know their existence (Hanken 1999; Crawford *et al.* 2010).

Because most conservation laws are based on species, an efficient means of identifying and discovering species and in particular highlighting potential complexes of multiple species is necessary. Such initiatives may involve new discoveries based on fieldwork, forensic applications based on specimens collected more than a century ago (Murphy *et al.* 2011), or simply more detailed examination of existing collections. Barcoding can provide an initial perspective on species diversity, which can be tested with other data sets to formulate or reinforce a sound taxonomic framework (Padial & De la Riva 2007). The task is not easy because taxonomy is expensive. The cost of describing a new species averages US $39,000–$122,000 including salaries (Carbayo & Marques 2011), although this sum can probably be reduced by a more effective use of bioinformatics, automated character recognition and molecular resources (Wheeler *et al.* 2012). For the sake of conservation alone, the identification and description of new taxa is of upmost importance for effective conservation planning and implementation. After all, bad taxonomy can lead to the demise of species (Daugherty *et al.* 1990; May 1990; Murphy *et al.* 2011). Standardized genetic markers, such as DNA barcodes, foster the twin goals of identification and description by clustering groups of specimens within and across life stages, highlighting divergent lineages and facilitating comparison of newly collected material with global databases (Vieites *et al.* 2009; Padial *et al.* 2010).

Identifying species once they have been described can also be problematic. In particular species complexes, whose resolution has relied on the use of advanced technologies, may be quite difficult to identify in field situations. Barcodes based on a sound systematic framework can in many cases provide the accuracy in identification needed at relatively low cost. Furthermore, sequencing of environmental DNA provides a reliable representation of amphibians present in aquatic habitats (Ficetola *et al.* 2008; Goldberg *et al.* 2011; Thomsen *et al.* 2012), allowing the establishment of fast monitoring protocols once a sequence database verified within a sound systematic framework with full taxon coverage is available. To improve our ability to efficiently deliver accurate identification outcomes for these important applications, we introduce Cold Code (http://www.coldcode.org), a global initiative to DNA barcode 'cold blooded' terrestrial vertebrates, the amphibians and reptiles.

Amphibians and reptiles combined comprise more than 16 200 nominal species. Independently, they rank third and fourth of the five vertebrate groups in terms of species diversity. In comparison, mammals contain about 6 000 species, birds about 10 000 species and fishes perhaps 30 000 species. As of September 21, 2012, Amphibia contains 7 036 species, of which 6 206 are frogs, 639 are salamanders and 191 caecilians (AmphibiaWeb 2012; accessed on 23 September 2012). The reptiles (The Reptile Database, numbers updated February 2012; Uetz *et al.* 2012) consist of 9 547 species. Among these are about 5 634 species of lizards, 181 amphisbaenians, 3 378 snakes, 327 turtles, 25 crocodilians and two tuataras (see also Zhang 2011). Numbers of described species are increasing in both amphibians and reptiles. For example, more than 3 000 species of amphibians have been described in the

last 25 years alone and, unlike for mammals or birds, there is no end to the discovery of new species in sight.

The task of DNA barcoding more than 16 000 species seems onerous, yet tissue samples already exist for more than 60% of the species (survey undertaken for the Genome 10K database; Haussler *et al.* 2009). *COI* barcoding of amphibians and reptiles started off at a turtle's pace with only few case studies using this gene (Hawkins *et al.* 2007; Smith *et al.* 2008; Vargas *et al.* 2009; Crawford *et al.* 2010; Naro-Maciel *et al.* 2010) in part because of methodological challenges caused by high mitochondrial DNA sequence variability, including PCR priming sites (Vences *et al.* 2005; Hawkins *et al.* 2007; Xia *et al.* 2012). However, recent advances in primer development (Che *et al.* 2012; Nagy *et al.* 2012; Table 1) are accelerating the initiative (Vences *et al.* 2012; Table 1); the new amphibian primers (Table 1) are working well for numerous species of Asian frogs and salamanders (Che *et al.* 2012) and also have been successfully tested in Malagasy mantellids (Vences *et al.*, unpublished data). To date, the Barcode of Life Data Systems (BoLD; http://www.boldsystems.org/) database holds almost 17 000 DNA barcoding records representing about 1 600 species of amphibians and approximately 8 000 records for about 1 500 species of reptiles (Table 2). These records represent independent initiatives and a summary of data mined from GenBank. Cold Code will not only accelerate the development of the open source database but also revise the taxonomic assignments of existing records in BoLD (Table 2).

Traditionally, many phylogenetic and phylogeographical studies of amphibians and reptiles used mitochondrial genes other than *COI* including *Cytb*, *ND2*, *ND4*, and especially 16S and 12S rRNA (e.g. Goebel *et al.* 1999). Several studies have used 16S as a complementary or sole barcoding marker, especially in amphibians, to link sequence data from new specimens to previously generated data (Vences *et al.* 2005; Vieites *et al.* 2009; Crawford *et al.* 2010). However, Xia *et al.* (2012) found that 16S did not identify species of salamanders as well as *COI*. For routine and high-throughput applications, and assuming a reference library of barcodes is available, *COI* barcodes will usually identify species of amphibians and reptiles. Although morphological data are indispensable for identifying specimens to the familial and generic levels, DNA barcodes often identify samples to the species level with higher speed and reliability than traditional morphological methods, for instance in larval amphibians (Strauß *et al.* 2010) or morphologically cryptic species of frogs (Vieites *et al.* 2009). Furthermore, the use of a single marker will facilitate environmental barcoding, which involves the detection of species using water-borne DNA only (Ficetola *et al.* 2008). Cold Code focuses on completing the COI database and we are considering the sequencing of 16S rRNA or other mitochondrial markers in select cases. Where possible, we will try to DNA barcode tissues of the same specimens used in multilocus phylogenetic studies to solidly link DNA barcode identifications to respective taxa in the tree of life.

Occasional mitochondrial introgression is well documented in amphibians and reptiles, causing divergent mitochondrial genomes to coexist within species (e.g. Babik *et al.* 2003; Shimada *et al.* 2008; Brown & Twomey 2009; Hauswaldt *et al.* 2011). In turtles, intergeneric hybrids occur naturally and in captivity, causing

Table 1 Aligned primers used in COI barcoding, comparing standard primers with new primers designed for amphibians and reptiles. Bold base pairs indicate degenerate nucleotides. dir = forward (F) or reverse (R) direction, (seq.) = primers used for sequencing

Primer seq 5′–3′	dir.	Name	Original taxon	Reference
GGT CAA CAA ATC ATA AAG A**Y**A T**Y**G G	F	dgLCO	Gastropods	Meyer *et al.* 2005;
GGT CAA CAA ATC ATA AAG ATA TTG G	F	LCO1490	Invertebrates	Folmer *et al.* 1994;
T**Y**T C**W**A C**W**A A**Y**C A**Y**A AAG A**Y**A TCG G	F	Chmf4	Frogs	Che *et al.* 2012;
A**Y**T CAA CAA ATC ATA AAG ATA TTG G	F	COI_C02	Salamanders, caecilians	Che *et al.* 2012;
T**Y**T C**W**A C**W**A A**Y**C A**Y**A AAG A**Y**A TTG G	F	COI_C01 (seq.)	Salamanders, caecilians	Che *et al.* 2012;
ATT CAA CCA ATC ATA AAG ATA T	F	LEP-F1	Lepidopterans	Hebert *et al.* 2004;
T**N**T T**M**T CAA C**N**A ACC ACA AAG A	F	RepCOI-F	Reptiles	Nagy *et al.* 2012;
T CAA CAA ACC A**Y**A AAG A**Y**A T**Y**G G	F	REPTBCF	Lizards	Castañeda & de Queiroz 2011;
TAA ACT TCA GGG TGA CCA AA**R** AA**Y** CA	R	dgHCO	Gastropods	Meyer *et al.* 2005;
TAA ACT TCA GGG TGA CCA AAA AAT CA	R	HC02198	Invertebrates	Folmer *et al.* 1994;
AC**Y** TC**R** GG**R** TG**R** CC**R** AA**R** AAT CA	R	Chmr4	Frogs	Che *et al.* 2012;
AC**Y** TC**R** GG**R** TGA CCA AAA AAT CA	R	COI_C04	Salamanders, caecilians	Che *et al.* 2012;
AC**Y** TC**Y** GG**R** TGA CCA AA**R** AA**Y** CA	R	COI_C03 (seq.)	Salamanders, caecilians	Che *et al.* 2012;
TAA ACT TCT GGA TGT CCA AAA A	R	LEP-R1	Lepidopterans	Hebert *et al.* 2004;
ACT TCT GG**R** TG**K** CCA AA**R** AAT CA	R	RepCOI-R	Reptiles	Nagy *et al.* 2012;
TAA ACT TCA GGG TGG CC**R** AA**R** AAT CA	R	REPTBCR	Lizards	Castañeda & de Queiroz 2011

Table 2 The number of *COI* DNA barcodes for species and specimens of amphibians and reptiles in the Barcode of Life Data Systems (BoLD; http://www.boldsystems.org/) (accessed 24 October 2012). Note that the taxonomy of many of these records requires revision, which will be carried out in the framework of Cold Code.

Higher taxon / family	Species and subspecies with barcodes	Specimens with barcodes
Amphibia		
Allophrynidae	1	4
Alytidae	10	154
Aromobatidae	27	132
Arthroleptidae	5	15
Bombinatoridae	13	106
Brachycephalidae	5	16
Brevicipitidae	3	5
Bufonidae	119	1033
Centrolenidae	34	250
Ceratophryidae	3	14
Craugastoridae	29	317
Cycloramphidae	10	29
Dendrobatidae	76	483
Dicroglossidae	71	855
Eleutherodactylidae	23	132
Heleophrynidae	0	2
Hemiphractidae	6	102
Hemisotidae	2	3
Hylidae	233	1565
Hyperoliidae	15	39
Leiopelmatidae	3	5
Leiuperidae	15	91
Leptodactylidae	80	618
Mantellidae	10	24
Megophryidae	39	107
Microhylidae	106	554
Myobatrachidae	56	1536
Pelobatidae	10	33
Pelodytidae	3	22
Petropedetidae	0	4
Phrynobatrachidae	3	12
Pipidae	23	195
Ptychadenidae	13	37
Pyxicephalidae	10	48
Ranidae	146	1105
Rhacophoridae	45	142
Rhinophrynidae	1	3
Sooglossidae	0	0
Strabomantidae	86	613
Ambystomatidae	24	139
Amphiumidae	3	4
Cryptobranchidae	4	11
Hynobiidae	65	317
Plethodontidae	64	402
Proteidae	4	7
Rhyacotritonidae	2	5
Salamandridae	104	470
Sirenidae	2	2
Caeciliidae	25	42
Ichthyophiidae	6	9
Rhinatrematidae	2	3
Total amphibians	1639	11816

Table 2 (Continued)

Higher taxon / family	Species and subspecies with barcodes	Specimens with barcodes
Crocodylia		
Crocodylidae	29	202
Lepidosauria		
Sphenodontidae	1	2
Acrochordidae	1	2
Agamidae	119	544
Amphisbaenidae	4	9
Anguidae	24	122
Aniliidae	1	2
Bipedidae	3	11
Boidae	19	169
Chamaeleonidae	75	141
Colubridae	296	953
Cordylidae	1	2
Corytophanidae	2	3
Cylindrophiidae	1	4
Dibamidae	1	1
Elapidae	9	39
Gekkonidae	231	683
Gerrhosauridae	14	16
Gymnophthalmidae	0	0
Helodermatidae	2	3
Hydrophiidae	0	1
Iguanidae	47	141
Lacertidae	122	577
Leptotyphlopidae	4	11
Liolaemidae	0	0
Phyllodactylidae	0	0
Polychrotidae	68	166
Scincidae	133	975
Sphaerodactylidae	0	0
Teiidae	6	6
Trogonophidae	1	2
Tropidophiidae	1	2
Typhlopidae	12	17
Varanidae	6	9
Viperidae	61	189
Xantusiidae	5	14
Xenopeltidae	1	3
Xenosauridae	1	3
Testudines		
Carettochelyidae	1	3
Chelidae	36	67
Cheloniidae	6	144
Chelydridae	4	19
Dermatemydidae	1	1
Dermochelyidae	1	16
Emydidae	41	65
Geoemydidae	58	196
Kinosternidae	22	34
Pelomedusidae	13	17
Podocnemididae	8	10
Testudinidae	33	64
Trionychidae	26	66
Total reptiles	1551	5726

taxonomic confusion (Fritz & Havaš 2007; Stuart & Parham 2007; Spinks *et al.* 2012). In such cases, nuclear gene barcode data are necessary to reliably identify species. Therefore, Cold Code is also considering the sequencing of a nuclear marker for certain taxonomic groups and geographical regions where advantageous.

Cold Code is being coordinated out of the Kunming Institute of Zoology (KIZ), Chinese Academy of Sciences. The administration of the initiative follows business standards. Co-chairs, R.W.M. and W.Z.W. report to the Steering Committee, which consists of the remaining authors. The steering committee sets policies and standards for the initiative, as well as seeks Coordinators who will take responsibility for particular groups, either by taxa or by region. For example, U.F. will coordinate efforts with turtles, and Z.T.N. will coordinate work on Africa. Volunteers are welcome; please inquire.

To assure success, the project is funded. In China, W.Z.W. oversees laboratory efforts. To bolster the initiative, Cold Code will sequence the first 10 specimens of a species at no cost to an investigator, but see below regarding cryptic species. Beyond this amount, sequencing will be done at cost of labour and expendables for those who contribute sequences to the initiative. As a policy, those requesting the service must submit relevant voucher data associated with the tissue samples. These data include but are not limited to species identification, collecting information locality (with GPS data), voucher specimen number, collector(s) and steward of the data. We recommend using the BoLD datasheet (see http://www.boldsystems.org/index.php/Resources/). Voucher images should also be submitted. KIZ will hold the data until either they are published, or for 3 years from date of generating the sequence data, whichever occurs first; other contributing facilities may have different guidelines. All data will go into BoLD as well as GenBank. Residual DNA will be banked securely at KIZ in the event that resequencing with new primers, verification or additional markers are needed. Residual tissue and DNA will not be distributed to any other researchers, including those at the host institution, without written permission of the steward. Upon request, Cold Code may cover shipping costs to China, and provide tissue tubes for those who cannot afford them on their own. Investigators with large collections of tissues and limited funding may be brought to China either for training or to gather the data on their own at the expense of Cold Code. Researchers with adequate funding may come to China and avail themselves of the facility, which has two 96-well ABI 3730XL automated sequencers plus several formats of next-generation sequencers.

For free sequencing, the accurate identification of species is critical. We conservatively estimate that museum collections on average suffer a 5% error rate in their databases. For counting species and taxonomy, Cold Code will rely on two online databases: Amphibian Species of the World (http://research.amnh.org/vz/herpetology/amphibia/) and the Reptile Database (http://www.reptile-database.org/). Whenever possible, specimens from the type locality should be submitted because of the great extent of cryptic genetic diversity and undescribed species (e.g. Vieites *et al.* 2009). Because of cryptic species, initially we will consider samples with >10% pairwise genetic distance to constitute a new species (candidate species; Vieites *et al.* 2009) for the purpose of providing the service.

Legal collecting and shipping is required. For fieldwork, the steward of the tissue should possess and retain the associated paperwork. Cold Code will not bank the permits. Presently, the shipment of CITES-controlled material to China is complicated. This sequencing may be done outside China or PCR product can be sent to KIZ for sequencing as it is exempt from CITES regulations; contact W.Z.W. for details.

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