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TECHNICAL NOTE

New primers for the amplification and sequencing of nuclear loci in a taxonomically wide set of reptiles and amphibians

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Abstract We report new primers for the amplification and sequencing of 11 nuclear markers in squamate reptiles and anuran amphibians (five in squamates, six in anurans). Ten out of the 11 loci are introns (three of which are linked) that were amplified using an exon-primed, intron-crossing (EPIC) PCR strategy, whereas an eleventh locus spans part of a protein-coding gene. Squamate and anuran primers were initially developed for *Lacerta schreiberi* (Squamata: Lacertidae) and *Pelodytes* spp. (Anura: Pelodytidae), respectively. Cross-species amplification of the squamate markers was evaluated in four genera representing two additional families, whereas for anurans three genera corresponding to three additional families were tested. Three out of the five loci were successfully sequenced in all squamate taxa tested. Cross-amplification of the six anuran markers had lower, but still significant, success. We predict these markers will be of great utility for both population genetics and phylogenetic studies.

Keywords Anura · Squamata · Nuclear sequence markers · Introns

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Reptiles and amphibians are becoming important model organisms for evolutionary studies. Their low dispersal capabilities and usually strong dependency on environmental conditions make these taxa particularly susceptible to climate change and appropriate models for studying the influence of climatic or geologic phenomena on the distribution of genetic diversity. Additionally, amphibians and reptiles are also amongst the most problematic animals in terms of conservation, with well documented world-wide declines in recent years (Stuart et al. 2004; Gibbons et al. 2000).

Most studies addressing evolutionary questions in amphibians and reptiles have relied solely on mitochondrial DNA (mtDNA) variation. MtDNA displays a set of properties that makes it a useful marker in phylogeographic and phylogenetic studies (Avise 2000); however, such single-locus studies are prone to erroneous interpretations, both because of random lineage sorting (e.g., Irwin 2002) and natural selection (e.g., Ballard and Whitlock 2004), and usually provide low resolution for the estimation of parameters of interest (Edwards and Beerli 2000). In recent years, the acknowledgement of these limitations and the improvement of analytical methods especially suited for multilocus data sets have led researchers to investigate the nuclear genome.

In reptiles and amphibians, however, this has proven to be a difficult task, since the pace of development of genomic resources (e.g. Expressed Sequence Tags (EST) databases, whole-genome sequencing projects, etc.) has been slow when compared to other vertebrate groups. For most cases, whenever primers for the amplification of nuclear genes were developed, these corresponded either to slow-evolving protein coding genes (e.g., Saint et al. 1998; Hoegg et al. 2004) with low utility for population genetics and phylogeography, or to species-specific efforts with low probability of cross-amplification in distant taxa (e.g., Dolman and Phillips 2004).

Here, we describe the development of nuclear sequence markers in several squamate reptiles and anuran amphibians occurring in a variety of regions including Southern Europe, North Africa, South America and Indian Ocean islands. The original target species of this work were Schreiber's green lizard (*Lacerta schreiberi*) and the Parsley frog (*Pelodytes ibericus/punctatus* group). *L. schreiberi* is an Iberian endemic species which comprises two well-defined phylogroups inferred from both mtDNA (4.67% divergence in cytochrome *b*; Paulo et al. 2001) and nuclear genealogies (Godinho et al. 2006). Iberian *Pelodytes* have been recently divided in two species (Sánchez-Herraíz et al. 2000) although mtDNA variation evidences the existence of four similarly distinct phylogroups showing 3.23–4.16% divergence in cytochrome *b* (M. Tejedo, personal communication).

Most of the loci we report were amplified following an exon-primed, intron-crossing (EPIC) polymerase chain reaction (PCR) methodology. We targeted genes for which mRNA sequences of either a reptile or an amphibian were available in GenBank. Resource databases (namely the *Ambystoma tigrinum* EST database—http://salamander.uky.edu—and the *Xenopus* genomic database—www.xenbase.org) were also used to choose putative target genes. For each gene our alignments typically further included human, mouse, chicken and zebrafish mRNA sequences (when available) and were visually inspected for highly conserved regions appropriate for primer anchorage. Whenever possible, primers were designed at least 50 bp away from the splicing site in order to ensure a sufficiently long exonic sequence for molecular confirmation of gene identity. For the locus *MC1R*, primers were designed based on an alignment of several squamate sequences obtained by Rosenblum et al. (2004), and chosen in order to amplify a fragment spanning ~700 bp of the gene.

A total of 24 markers was tested in 2–5 individuals of both *L. schreiberi* and *Pelodytes* spp. Initial essaying conditions were the same for all the markers and for both species, although some adjustments had to be made prior to sequencing: PCRs were carried out in 10 µl volume containing 1× PCR buffer (50 mm Tris–HCl, 50 mm NaCl, pH 8.5); 3 mM $MgCl_2$; 0.4 mM each dNTPs, 0.5U of GoTaq DNA polymerase (Promega), 0.3 µM each primer and approximately 50 ng of genomic DNA. Amplification conditions consisted of a pre-denaturing step of 5 min at 92°C followed by 40 cycles of a denaturing step of 30 s at 92°C, annealing at 50°C for 30 s and extension at 72°C for 90 s. The final extension was accomplished at 72°C for 5 min. For successful amplifications, PCR products were enzymatically purified (with the ExoSap-IT purifying kit, Amersham-Pharmacia Biotech) and sequenced from both ends using BigDye 3.1 on an Applied Biosystems 3100xl DNA sequencer.

From the tested markers, five (*CTSDint8*, *MC1R*, *NFYCint16*, *PKM2int5* and *RELNint61*) were successfully amplified and sequenced in *L. schreiberi*, and another four (*CHERPint7*, *PPPCAint4*, *RPL3int5* and *RPL9int5*) had similar success in *Pelodytes*. Two additional loci, corresponding to introns 4 and 6 of the *RPL9* gene (and hence in close linkage to *RPL9int5*) were also amplified in the latter species, but only with marginal success (most individuals did not amplify). From these two markers, good quality sequences were obtained only for *RPL9int4*.

The sequences of the primers developed in this study are presented in Table 1.

Cross-amplification tests for the loci developed for *L. schreiberi* were performed in *Podarcis* (Lacertidae; species used: *P. bocagei* and *P. carbonelli*, 12.6% divergence in cytochrome *b* (Pinho et al. 2006)), *Mabuya* (Scincidae; species used: *M. seychellensis* and *M. wrightii*, ~8% divergence in cytochrome *b* (S. Rocha, unpublished data)), *Phelsuma* (Gekkonidae; species used: *P. sundbergi* and *P. astriata*, ~13% divergence in cytochrome *b* (S. Rocha, unpublished data)) and *Urocotyledon inexpectata* (Gekkonidae; two intraspecific phylogroups showing a divergence of ~8% in cytochrome *b* (S. Rocha, unpublished data)). For anurans, we essayed cross-amplification of *Pelodytes* primers in *Alytes* (Discoglossidae; taxa used: *A. cisternasii*, *A. maurus*, *A. dickhilleni*, *A. muletensis* and 4 subspecies of *A. obstetricans*, with divergence ranging from 0.29 to 15.5% for cytochrome *b* (Martínez-Solano et al. 2004)), *Rhinella marina* (Bufonidae; three phylogroups with divergences ranging from 1.2 to 2.3% in cytochrome *b* and control region combined (M. Vallinoto, unpublished data)) and *Phyllomedusa* (Hylidae; species used: *P. burmeisteri* and *P. iheringii* showing a divergence of 7.7% in ND2 (T. O. Brunes, unpublished data)). We used the initially developed amplification protocol with minor modifications for each species.

Success in amplification and sequencing was obtained for all five squamate markers in *Podarcis*, in four markers (all but *CTSDint8*) in the two gekkonid genera and three markers in *Mabuya* (*MC1R*, *PKM2int5* and *RELNint61*). In contrast, in anurans almost non-overlapping subsets of the

Table 1 Primers successfully used in this study for the amplification of nuclear markers

Group	Gene	Intron[a]	Locus abbreviation	Primer name	Primer utility[b]	Primer sequence 5′-3′
Squamates	Cathepsin D	8	*CTSDint8*	CTSD8F	A	TACATGCTTCCATGTGATAAGCTGT
				CTSD9R	A	ATGAAGACATCTCCCAGGATCCA
	Melanocortin receptor 1	–	*MC1R*	MC1RF	A	GGCNGCCATYGTCAAGAACCGGAACC
				MC1RR	A	CTCCGRAAGGCRTAAATGATGGGGTCCAC
	Nuclear transcription factor Y, gamma	16	*NFYCint16*	NFYC16F1	B	GTCCAGCARGGACAGCAGCAGTTCAGC
				NFYC16F2	C	GCARGGACAGCAGCAGTTCAGCCAGTT
				NFYC17R1	D	GGCATRGTSACTTGCTGRATCTGGTA
				NFYC17R2	E	GCWGGCATRGTSACTTGCTGRATCTGG
				NFYCLscF	F	GTTGGATTAAAAGAGGATCAGACA
	Pyruvate kinase, muscle	5	*PKM2int5*	PKM25F	G	AGTGGYACAGCAGARGTGGAGCTCAA
				PKM26R	G	TTYTCAATYTTGCTGATRATCTTGATG
				PKSQF	A	ACCAAAGTTGTWGATGTTGGCAGC
				PKSQR	A	ATGAAGGAAGCAAACACCATGTC
	Reelin	61	*RELNint61*	RELN61F	A	GAGTMACTGAAATAAACTGGGAAAC
				RELN62R	A	GCCATGTAATYCCATTATTTACACTG
Anurans	Calcium homeostasis endoplasmic reticulum protein	7	*CHERPint7*	CHERP7F1	H	GCTCTGGGARAAGAAYGGCTACTT
				CHERP7F2	I	CTGGGARAAGAAYGGCTACTTYGATGA
				CHERP8R	J	CTGNAKCTGCTGCTGGAAKGCCA
	Protein phosphatase 3, catalytic subunit, alpha isoform	4	*PPP3CAint4*	PPP3CA4F1	K	CTGTAYTTGTGGGCCTTGAAAATTC
				PPP3CA4F2	G	CTWCGTGGRAATCATGAATGTAGACAT
				PPP3CA5R1	L	AAGGCATCCATGCAGGCATCATATA
				PPP3CA5R2	H	GGCAGTCAAAGGCATCCATGCAGGC
				PPP3CAPelF	I	CGTGGGAATCATGAATGTAGACATCT
	Ribosomal protein L3	5	*RPL3int5*	RPL35F	M	AAGAAGTCYCACCTCATGGAGAT
				RPL36R	J	TTRCGKGGCAGTTTCTTTGTGTGCCA
				RPL36RA	N	AGTTTCTTTGTGTGCCAACGGCTAG
				RPL3intF	P	AGTCTTTGGCCAGGATGAAATG
				RPL3intR	P	TCACACCTAGGAGGGATAATG
	Ribosomal protein L9	4	*RPL9int4*	RPL94F	O	CGTGTKGACAAATGGTGGGGTAA
				RPL95R	O	ATGGGAAAGTGAGCRTACACAGA
		5	*RPL9int5*	RPL95F	J	TCTGTGTAYGCTCACTTTCCCAT
				RPL96R	J	AGAATCAGYTCRTCTTTCTGGGCTTG
		6	*RPL9int6*	RPL96F	J	CAGAAAGAYGARCTGATTCTTGAAGG
				RPL97R	J	TACWGTGGTGGCYTGCTGGATCAAGG
				RPL96intF	N	TGTACAGGTCAAGTGTTATC
				RPL96intR	N	ATGCCAGTTAAAAATCAGACC

[a] Intron number is based on the nomenclature used in humans

[b] Legend for primer utility: *A* used for routine amplification and sequencing in all squamates, *B* used for amplification only in *Phelsuma* and *Urocotyledon* (also amplifies *Lacerta* and *Podarcis* but was not used routinely), *C* used for routine amplification in *Lacerta* and *Podarcis* and for sequencing in *Urocotyledon* and *Phelsuma*, *D* internal primer used only for sequencing, *E* used for amplification, *F* internal primer used for sequencing in *Lacerta* and *Podarcis*, *G* used for some amplifications and sequences, but not routinely, *H* used for amplification only (in *Pelodytes*), *I* used only for sequencing in *Pelodytes* (also amplifies), *J* used for both amplification and sequencing in *Pelodytes*, *K* used for amplification only in *Pelodytes* and for both amplification and sequencing in *Alytes*, *L* used only for sequencing in *Pelodytes* and for both amplification and sequencing in *Alytes*, *M* used for amplification and sequencing in *Pelodytes* and *Rhinella*, *N* used routinely for amplification and sequencing in *Rhinella*, *O* used routinely for amplification and sequencing in *Pelodytes* and *Alytes*, *P* used routinely for amplification and sequencing in *Phyllomedusa*

markers developed for *Pelodytes* were amplified in other genera: *PPP3CAint4* and *RPL9int4* in *Alytes*, *RPL3int5* in *Rhinella* and *Phyllomedusa*, and *RPL9int6* in *Rhinella*. In some cases, in order to optimize amplification and/or sequencing, new primers, specific for the genus in question, were designed (also reported in Table 1).

Table 2 Summary statistics for the markers developed in this study

Group	Genus	Marker	*N* (per species or phylogroup)[a]	Length (bp)	*S*	π	*h*	Hd	Rm	Indels (size)
Squamates	*Lacerta*	*CTSDint8*	9 (5/4)	688–689	6	0.00177	7	0.793	0	1 (M)
		MC1R	10 (6/4)	614	3	0.00185	3	0.416	0	0
		NFYCint16	12 (4/8)	903–919	10	0.00387	7	0.793	1	1 (16)
		PKM2int5	12 (7/5)	376–401	2	0.00065	3	0.236	0	2 (23/2)
		RELNint61	17 (9/8)	721–723	10	0.00559	6	0.738	1	1 (2)
	Podarcis	*CTSDint8*	10 (5/5)	688–695	30	0.01423	14	0.968	9	4 (1/6/4/2)
		MC1R	9 (6/3)	649	18	0.00677	10	0.876	2	0
		NFYCint16	5 (5/0)	797	3	0.00196	4	0.778	1	0
		PKM2int5	8 (5/3)	352	12	0.00999	10	0.942	1	0
		RELNint61	7 (4/3)	860–897	34	0.01034	11	0.967	3	6 (12/1/22/M^b/M^b/1)
	Mabuya	*MC1R*	10 (5/5)	633	15	0.01148	6	0.805	1	0
		PKM2int5	13 (13/0)	418	3	0.00169	3	0.514	0	0
		RELNint61	10 (5/5)	914–921	11	0.00394	7	0.832	0	5 (7/5/11/3/1)
	Urocotyledon	*MC1R*	10 (5/5)	621	6	0.00305	8	0.836	1	0
		NFYCint16	6 (2/4)	220	17	0.02624	9	0.955	3	0
		PKM2int5	5 (2/3)	735–736	9	0.00577	5	0.822	1	1 (1)
		RELNint61	10 (5/5)	813–816	39	0.00950	18	0.989	4	4 (1/2/2/1)
	Phelsuma	*MC1R*	10 (5/5)	617	13	0.00715	12	0.937	2	0
		NFYCint16	5 (4/1)	172	2	0.00329	3	0.511	0	1 (M^b)
		PKM2int5	10 (5/5)	520–521	14	0.00673	10	0.837	0	1 (1)
		RELNint61	10 (5/5)	875–877	24	0.00613	14	0.947	1	3 (M^b/1/1)
Anurans	*Pelodytes*	*CHERPint7*	8 (3/2/1/2)	676–698	13	0.00875	5	0.808	0	2 (1/22)
		PPP3CAint4	14 (5/4/3/1)	956–1,428	34	0.01085	10	0.881	0	11 (17/115/23/20/288/M^b/22/1/7/4/1)
		RPL3int5	9 (2/2/3/2)	636–639	20	0.00959	10	0.902	1	2 (1/2)
		RPL9int4	3 (1/1/0/1)	660–681	15	0.00835	3	0.733	0	8 (11/2/2/4/1/1/2/4)
		RPL9int5	8 (2/2/3/1)	571–575	14	0.01019	7	0.75	2	2 (2/2)
	Alytes	*PPP3CAint4*	8 (1/1/1/1/1/1/1/1)	691–700	68	0.02700	9	0.942	2	11 (4/1/4/4/2/3/1/2/7/1/1)
		RPL9int4	8 (1/1/1/1/1/1/1/1)	491–497	26	0.01873	8	0.933	2	8 (1/1/1/2/2/M^b/4/5)[c]
	Rhinella	*RPL3int5*	8 (2/6/0)	673–676	6	0.00370	5	0.775	0	1 (3)
		RPL9int6	6 (2/3/1)	497–501	12	0.00924	6	0.848	0	1 (4)
	Phyllomedusa	*RPL3int5*	5(3/2)	530–532	33	0.02935	6	0.889	0	1 (2)

From left to right: *N*, number of individuals analysed in this study; Length, size of the sequences in the final alignment used; *S* number of segregating sites, π nucleotide diversity, *h* number of haplotypes, *Hd* nucleotide diversity, *Rm* minimum number of recombination events, *Indels* number of insertion/deletion polymorphisms found. Summary statistics were calculated excluding indels

[a] The number of individuals per phylogroup or species is represented in parenthesis; these correspond to phylogroups in the case of *L. schreiberi*, *U. inexpectata*, *Pelodytes* and *R. marina* and distinct species in all other cases, except *Alytes* where both distinct species and subspecies within *A. obstetricans* were analysed

[b] *M* means a repeat region consisting of the repetition of a single base and where actual indel length was variable across the data set; we consider two such motifs in tandem as a single repeat region. Because in these regions it is difficult to evaluate repeat number, the reported sequence length considers only the larger apparent size for the repeat region observed

[c] This locus also included one inversion spanning 25 bp

After preliminary amplification and sequencing essays, we resequenced at least five individuals from each genus in order to evaluate diversity levels of the newly developed markers across taxa. All the sequences generated for this study were submitted to GenBank (accession numbers GU180920–GU181194). We used both statistical and laboratorial procedures to determine haplotype phase: for a first assessment, we used the Bayesian algorithm implemented in the software PHASE v2.1 (Stephens et al. 2001). Runs consisted of 1,000 iterations of burn-in and 1,000 main iterations, with a thinning interval of 1. For each data set, we repeated the runs five times with different random

seeds. We used a threshold of 0.80 posterior probability to accept a given reconstruction. Second, for a subset of the samples for which PHASE could not reconstruct haplotypes, PCR fragments were cloned using the pGEM-T-Easy Vector Systems kit (Promega). A minimum of 5 clones per sample were sequenced. Finally, we re-ran PHASE using the information from the reconstructed haplotypes. Phased data sets were trimmed and aligned by eye using BioEdit v7.0.9.0 (Hall 1999) and imported to DNAsp v5.00.7 (Librado and Rozas 2009) for polymorphism analysis. We calculated standard summary statistics and the minimum number of recombination events (Rm, Hudson and Kaplan 1985) (see Table 2).

All the markers, even short introns, showed polymorphism in every genus, with some particular genes showing remarkably high levels of variability. We may conclude that the markers we developed can be used within a broad taxonomic scope. Moreover, they offer substantial polymorphism levels for both population genetics and phylogenetic approaches. We expect that these markers can therefore provide an important tool for the study of the evolutionary history and conservation genetics of reptiles and amphibians.

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