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A combination of techniques proves useful in the development of nuclear markers in the newt genus *Triturus*

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Abstract

To increase the number of markers available for study of phylogeny and phylogeography in the newt genus *Triturus*, we developed and tested 59 primer pairs using three different techniques. Primers were obtained from published sources, by designing exon-primed intron-crossing primers and from randomly cloned anonymous nuclear DNA fragments. Successful polymerase chain reaction products were cloned and sequenced. Five fragments were successfully amplified and sequenced for six species of *Triturus*: intron 7 of the β-fibrinogen gene (βfib*int*7), third intron of the calreticulin gene (Cal*int*C), the 11th intron of the α-subunit of the platelet derived growth factor receptor (PDGFRα) and two anonymous markers (Cri1 and Cri4). The average percentage species divergence across all the markers is low (*c*. 3%), compared to what has been found in mitochondrial DNA (25–30%).

Keywords: Amphibia, anonymous markers, introns, newts, nuclear DNA markers, *Triturus*

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Mitochondrial DNA (mtDNA) has been the prime tool used in both phylogenetic and phylogeographical studies due to its abundance in the cell, uniparental inheritance and (mostly) nonrecombining nature. This translates into a relatively straightforward accessibility and availability of established analytical techniques (Avise 1994). In recent years, however, the wisdom of relying on only this molecule for drawing evolutionary inferences at the inter- and intraspecific levels have been repeatedly questioned (for example, Ballard & Whitlock 2004). Even if multiple mtDNA regions are studied, they do not provide independent

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Table 1 Primers that successfully produced PCR bands and sequences in the genus *Triturus*

Gene	Symbol	Primer	Sequence 5′–3′	*N*	Length (bp)	SNPs	Number of indels (length — bp)	π	H_d	H_O
β-fibrinogen intron 7	βfib*int7*	FIBX7[1]	GGAGANAACAGNACNATGACAATNCAC	25	500–514	38	1 (14)	0.022	0.960	0.56
		FIBX8[1]	ATCTNCCATTAGGNTTGGCTGCATGGC							
		BFXF[1]	CAGYACTTTYGAYAGAGACAAYGATGG							
		BFXR[1]	TTGTACCACCAKCCACCRTCTTC							
		BF CRI 1F	AAGTAGTGCTCCAGGCTTCATC							
		BF CRI 1R	GCACACTGTGTTAATCCTCCTG							
Platelet-derived growth factor receptor α	PDGFRα	PDGFRa F[2]	CGGGTCATTGAGTCCATCAGCC	24	617–662	66	9 (1, 12, 4, 8, 3, 1, 13, 2, 1)	0.031	0.978	0.50
		PDGFRa R[2]	CAGTGGGTTTTAACATTTTCACAG							
		PDGFRa Fa	GTCATTGAGTCCATCAGCCCTG							
		PDGFra 2F	AGCTGCCCTATGACTCCAGATG							
		PDGFra 2R	GCTCAAGCCATACGCTGTTCCT							
		PDGFra 2Ra	GCTCAAGCCATACGCTGTTCCTTC							
Calreticulin intron C	Cal*int*C	CalC 1F	GGMGACTCAGARTACAACATCAT	21	461–509	39	8 (1, 1–2, 2, 3, 3, 6–22, 3, 10)	0.026	0.957	0.31
		CalC 1Rb	GAATGTCYTTGTTGATCTGCATGT							
		CalC 3F	CGTTTGCGTCCAGTGTATTG							
		CalC 3R	GTCGGAGGTCCGCAGATGT							
		CalC 4R	GTCCTTGTTGATCTGCAGGTTT							
Cristatus anonymous locus 1	CRI1	CRI1 1F	ATCGCGACTGGGAGTCTTATT	24	493–511	51	5 (1, 1–2, 1, 1, 11–12)	0.025	0.976	0.50
		CRI1 1R	ATGTTCTATGCCCTCCCAGAGT							
		CRI1 1Fa	GCGACTGGGAGTCTTATTTCG							
		CRI1 1Ra	GTTCTATGCCCTCCCAGAGTGTG							
Cristatus anonymous locus 4	CRI4	CRI4 1F	AGCTCTTTGAAGACAGCATTCC	19	507–512	38	4 (2, 1, 1, 1)	0.024	0.942	0.19
		CRI4 1R	CGCTTTGTGAACTACCATACCA							
		CRI4 1Fb	CTCTTTGAAGACAGCATTCCAG							

Number of chromosomes screened (*N*), the length of amplified fragments, the number of single nucleotide polymorphism (SNPs), the number and length of indels in the alignment, nucleotide (*p*) and haplotype diversity (H_d) and observed heterozygosity (H_O).
References: [1], Sequeira *et al*. (2006); [2], Voss *et al*. (2001).

information due to the lack of recombination at this molecule. Moreover, because individual loci in the genome have different histories, reflecting stochasticity of the coalescent process, multiple loci are essential for reconstructing historical evolutionary processes within species (for example, Felsenstein 2006). The sampling of multiple unlinked loci will, by averaging out genealogical stochasticity, provide better estimation of population parameters which are usually the values of interest. For 'nonmodel' species, however, nuclear sequence markers are often unavailable. Genomic data from various genome projects provide information that can be employed for tackling problems in other species. The focus of our attention is the newt genus *Triturus*. These newts have huge genomes (10 times bigger than the human genome; Gregory *et al*. 2007) and using prior information is the genetic equivalent of using a magnet to find a needle in a haystack.

Triturus is a group of closely related species of newts (Salamandridae: Amphibia). It includes the members of the Crested newt group (*Triturus cristatus* superspecies) and the Marbled newt group (*Triturus marmoratus* species pair). The divergence of the group is estimated at a minimum of 24 million years ago (Steinfartz *et al*. 2007). The level of mtDNA genetic differentiation is around 10% for the crested newts (Arntzen *et al*. 2007), 5% for the marbled newts (G.E.T. and J.W.A., unpublished results) and 25–30% for the genus as a whole (Steinfartz *et al*. 2007).

We here describe the development of nuclear sequence markers for the study of the genus *Triturus* through three different strategies. First, we tested published primers known to work in other salamanders, amphibians in general or fishes. Second, we developed exon-primed intron-crossing (EPIC) primers. Searching databases such as GenBank, we downloaded relevant sequences and designed primers in conserved regions of adjacent exons, close to the intron–exon boundaries. And third, we focused on anonymous markers, that is, random sequences of nuclear DNA from an unknown location in the genome. To obtain these, we cloned unspecific bands co-amplified in other polymerase chain reactions (PCR) and then checked the sequences for single base repeats and base diversity. Note that we did not, as is more usual, construct a genomic library (Karl & Avise 1993; Jennings & Edwards 2005).

Fifty-nine primer pairs were tested through PCR and sequencing (see Table S1, Supporting information). If PCRs yielded multiple bands, those of similar size were cut from the gel and purified using the QIAGEN gel extraction kit (QIAGEN) before cloning. Successful first-round PCR products

were cloned with the pGEM-T easy cloning kit (Promega). Plasmid DNA was extracted from overnight cultures of individual colonies and inserts were sequenced in both direc-tions. The criteria to select fragments were size (> 500 bp), the absence of large repeats, the amount of genetic variation as well as PCR and sequencing efficiency.

The sequences obtained were compared for similarity to sequences deposited in GenBank using the BLAST algorithm (Altschul *et al*. 1990). Except for the anonymous markers, the external fragments matched the exon regions (adjacent to exon/intron boundaries) of the respective genes whereas the anonymous markers did not show any BLAST hits with GenBank. Based on the new sequences, primers were then redesigned, to increase PCR efficiency and specificity and PCR products so obtained were sequenced directly. The basic PCR programme consisted of 4 min at 95 °C, followed by 35 cycles of successive denaturing (95 °C) for 30 s, annealing (57–68 °C depending on the fragment) for 30 s and extension (72 °C) for 90 s, and a final extension (72 °C) of 3 min. Reaction chemistry was 23 μL of H_2O, 3 μL of buffer (15 μM $MgCl_2$), 1.8 μL of 25 μM $MgCl_2$, 0.6 μL of dNTPs (10 mM), 0.2 μL of each primer (100 μM) and 0.2 μL (1 U) of *Taq* DNA Polymerase (QIAGEN).

Sequences were obtained from intron 7 of the β-fibrinogen gene (βfib*int*7), intron C of the calreticulin gene (Cal*int*C), intron 11 of the α-subunit of the platelet derived growth factor receptor (PDGFRα*int*11) and for two anonymous markers (Cri1 and Cri4). Several sequences displayed length size polymorphisms or single nucleotide polymorphisms(SNP). To resolve length size polymorphisms, we read the unphased chromatogram by eye comparing it to homozygous sequences from other individuals of the same species. For sequences with more than one SNP, we used Phase version 2.1 (Stephens *et al*. 2001; Stephens & Donnelly 2003) to reconstruct the haplotypes. Sequences are available from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) with accession nos FJ526219–FJ526331. Polymorphisms for these five fragments are described in Table 1. The genes were successfully amplified for all six species of *Triturus*. Average percentage species divergence was *c*. 3% for the genus and *c*. 1% for the groups of crested newt species and marbled newts, respectively.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Primer pairs tested for usability in the genus *Triturus*. Published primers were retrieved from the literature, EPIC (Exon-Primed Intron-Crossing) primers were designed based on GenBank sequences of related groups and anonymous markers were based on sequences from unspecific PCR bands (see text for details). Primers in bold were selected for further testing

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