Newts in time and space: the evolutionary history of Triturus newts at different temporal and spatial scales

Espregueria Themudo, G.

CHAPTER 3

A COMBINATION OF TECHNIQUES PROVES USEFUL IN THE DEVELOPMENT OF NUCLEAR MARKERS IN THE NEWT GENUS *TRITURUS*

Espregueira Themudo, G.[1,2], W. Babik,[3,4] & J.W. Arntzen[1]

[1] *National Museum of Natural History Naturalis, PO Box 9517, 2300 RA Leiden, the Netherlands*
[2] *CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal*
[3] *Helmholtz Centre for Environmental Research - UFZ, Department of Community Ecology, Theodor-Lieser-Strasse 4, 06120 Halle (Saale), Germany*
[4] *Institute of Environmental Research, Jagiellonian University, Gronostajowa 7, 30-387 Krakow, Poland*

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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 (EPIC) and from randomly cloned anonymous nuclear DNA fragments. Successful PCR 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 eleventh 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*.

Main text

Mitochondrial DNA has been the prime tool used in both phylogenetic and phylogeographic studies due to its abundance in the cell, uniparental inheritance and (mostly) non-recombining 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 intra-specific levels have been repeatedly questioned (for example, BALLARD and WHITLOCK, 2004). Even if multiple mtDNA regions are studied, they do not provide independent 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 'non-model' 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 (ten 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 hay-stack.

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 Ma (million years before present; 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 (GET and JWA, 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. Firstly, we tested published primers known to work in other salamanders, amphibians in general or fishes. Secondly, we developed Exon-primed Intron-crossing (EPIC) primers. Searching databases such as GenBank we downloaded relevant sequences and designed primers in conserved

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).

regions of adjacent exons, close to the intron-exon boundaries. And thirdly, we focussed on anonymous markers, i.e., random sequences of nuclear DNA from an unknown location in the genome. To obtain these we cloned unspecific bands co-amplified in other PCR's 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 (JENNINGS and EDWARDS, 2005; KARL and AVISE, 1993).

Fifty-nine primer pairs were tested through PCR and sequencing (see Supplementary information). If PCRs yielded multiple bands, those of similar size were cut from the gel and purified using the Qiagen gel extraction kit (Qiagen) prior to 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 directions. The criteria to select fragments were size (>500 bp), the absence of large repeats, the presence of sufficient 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 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 program consisted of four minutes at 95 ºC, followed by 35 cycles of successive denaturing (95 ºC) for 30'', annealing (57-68 ºC depending on the fragment) for 30'' and extension (72 ºC) for 90'', and a final extension (72 ºC) of three minutes. 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 eleven of the α-subunit of the platelet derived growth factor receptor (PDGFRα*int11*) 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 v.2.1 (STEPHENS and DONNELLY, 2003; STEPHENS *et al.*, 2001) to reconstruct the

haplotypes. Sequences are available from GenBank (http://www.ncbi.nlm.nih.gov/Genbank) with accession numbers 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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Appendix 1 - 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.

Gene	Forward Primer	sequence 5' - 3'	Reverse Primer	sequence 5' - 3'	Reference
Published primers					
1H3	1H3F	GGC AAA TGC TGG TCC CAA CAC AAA	1H3R	CGA CAA CAC TGC CAA ATA CCA CAT	Putta *et al.* (2005)
Beta-fibrinogen intron 7 (βfib*int7*)	**FIBX7**	**GGA GAN AAC AGN ACN ATG ACA ATN CAC**	**FIBX8**	**ATC TNC CAT TAG GNT TGG CTG CAT GGC**	**Sequeira *et al.* (2006)**
Beta-fibrinogen intron 7 (βfib*int7*)	**BFXF**	**CAG YAC TTT YGA YAG AGA CAA YGA TGG**	**BFXR**	**TTG TAC CAC CAK CCA CCR TCT TC**	**Sequeira *et al.* (2006)**
Beta-fibrinogen intron 7 (βfib*int7*)	**BF-CRI-1F**	**AAG TAG TGC TCC AGG CTT CAT C**	**BF-CRI-1R**	**GCA CAC TGT GTT AAT CCT CCT G**	**this study**
c-myc	cmyc1U	GAG GAC ATC CTG GAA RAA RTT	cmyc3L	GTC TTC CTC TTG TCR TTC TCY TC	Crawford (2003)
Collagen 1a	Col1a1-f	CAC CGA AGC CTC CCA AAA CAT CAC	Col1a1-r	GAG CCC TTC CAT CTT AGT CGT	Voss *et al.* (2001)
Homeo box a4 (HoxA4)	HoxA4-f	CTG CAG CAC TGG CAG GTC CTG CTG	HoxA4-r	TGG CGA GCG CAT CTT GGT GTT GG	Voss *et al*. (2001)
Platelet-derived growth factor receptor *alpha* (PDGFRα)	**PDGFRA f**	**CGG GTC ATT GAG TCC ATC AGC C**	**PDGFRA r**	**CAG TGG GTT TTA ACA TTT TCA CAG**	**Voss *et al.* (2001)**
Recombination activating gene 1 (Rag-1)	Mart FL1	AGC TGG AGY CAR TAY CAY AAR ATG	Amp-RAG1 R1	AAC TAC GCT GCA TTK CCA ATR TCA CA	Chiari *et al.* (2004)
Recombination activating gene 1 (Rag-1)	Amp F2	ACN GGN MGN CAR ATC TTY CAR CC	Amp-RAG1 R1	AAC TAC GCT GCA TTK CCA ATR TCA CA	Chiari *et al.* (2004)
Recombination activating gene 1 (Rag-1)	Amp-RAG1 F1	ACA GGA TAT GAT GAR AAG CTT GT	Mart R6	GTG TAG AGC CAR TGR TGY TT	Chiari *et al.* (2004)
Recombination activating gene 1 (Rag-1)	Amp-RAG1 F1	ACA GGA TAT GAT GAR AAG CTT GT	Amp R2	GGT GYT TYA ACA CAT CTT CCA TYT CRT A	Chiari *et al.* (2004)
Recombination activating gene 2 (Rag-2)	Rag-2A-F35	TGG CCN AAA MGN TCY TGY CCM ACW GG	Rag2.Lung.320R	AYC ACC CAT ATY RCT ACC AAA CC	Hoegg *et al.* (2004)
Recombination activating gene 2 (Rag-2)	Rag-2.Lung.35F	GGC CAA AGA GRT CYT GTC CNA CTG G	Rag2.Lung.320R	AYC ACC CAT ATY RCT ACC AAA CC	Hoegg *et al.* (2004)
Recombination activating gene 2 (Rag-2)	31 FN. Venk	TTY GGN CAR AAR GGN TGG CC	Lung.460R	GCA TYG RGC ATG GAC CCA RTG NCC	Chiari *et al.* (2004)
Rhodopsin	Rhod.ma	AAC GGA ACA GAA GGY CC	Rhod.md	GTA GCG AAG AAR CCT TC	Hoegg *et al.* (2004)
Rhodopsin (Rho)	Rho-f	CCA AGA GTT CTG CCA TCT ACA ATC CAG	Rho-r	CGC AGG AGA AAC CTG GCT GGA AGA CAC	Voss *et al.* (2001)
v-kit (KIT)	Kit f	TCC GTG TGG GAA TCC AGT CAC T	Kit r	AGA TGG CAT ATC TGG GAC ATA TTC	Voss *et al*. (2001)
EPIC primers					
Aldolase C (Ald) intron C	AldC2F	GGT GGA AAA CAC AGA GGA GAA C	AldC1R	CCA GAG GAA CGA CAC CTT TAT C	this study
Aldolase C (Ald) intron D	AldD1F	TTG ATA AAG GTG TCG TTC CTC TG	AldD1R	CTC ACT GAT CTT CAG CAC ACA AC	this study
Aldolase C (Ald) intron F	AldF1F	CTG ATG GAG ACC ATG ACY TGA A	AldF1R	ATG GCA ATC TCC TCA GGA CTG TA	this study
Aldolase C (Ald) intron G	AldG1F	GAC GCA CTG TAC CAC CTG CT	AldG1Ra	AGA GCA TTT GTT GAT GGC ATT C	this study
Aromatase P450	P450F	GAA ATA TTG AAC CCC ATG CAC TA	P450R	CCT GGT ATT GTT GAC GTT TCT TC	this study
Calreticulin (Cal) intron C	**CalC1F**	**GGM GAC TCA GAR TAC AAC ATC AT**	**CalC1Rb**	**GAA TGT CYT TGT TGA TCT GCA TGT**	this study
Calreticulin (Cal) intron C	**CalC3F**	**CGT TTG CGT CCA GTG TAT TG**	**CalC3R**	**GTC GGA GGT CCG CAG ATG T**	this study
Calreticulin (Cal) intron C	**CalC3F**	**CGT TTG CGT CCA GTG TAT TG**	**CalC4R**	**GTC CTT GTT GAT CTG CAG GTT T**	this study
Elongation Factor (EF) intron C	EFC1F	ACA TCA AGA AAA TCG GCT ACA AC	EFC1R	ATT TCC CTC CTT ACG GTC AAC	this study
Elongation Factor (EF) intron C	EFC1F	ACA TCA AGA AAA TCG GCT ACA AC	EFC2R	CAC TGG CAT TTC CCT CCT TMC	this study
Elongation factor alpha, intron E	EFE2F	GGT GAG TTG AGT GTT GCG TTT A	EFE3R	GAC CAG GGT GAT TCA GAA TAA TG	this study
Glyceraldehyde-3-phosphate dehydrogenase (Gapd) intron B	GapdB1F	AAG ATG AAA GTA GGA GTC AAT GG	GapdB1R	GAC TAC AGC GCG GGT CAC	this study
Glyceraldehyde-3-phosphate dehydrogenase (Gapd) intron B	GapdB1F	AAG ATG AAA GTA GGA GTC AAT GG	GapdB2R	AGT TGA CTA CAG CGC GGG TCA C	this study
Glyceraldehyde-3-phosphate dehydrogenase (Gapd) intron D	GapdD1F	CTG AGA ACG GCA AAC TTG TMA TC	GapdD1Ra	TTT GTC AAT GGT GGT GAA CAC T	this study
Growth Hormone (GH) intron C	GHC1Fa	ACA GCA TTC TGC TGC TCT GA	GHC1Ra	AGA CCG AAT GAG AGT CAA RGA GA	this study
Growth Hormone (GH) intron D	GHD1F	CTA CGA GAG GCT TAA GGA CYT GG	GHD1R	GTC TTT CTT GAA GCA GGA TAG CA	this study
Growth Hormone (GH) intron D	GHD2F	CAC ATG AGA TTC TTT CCC CAG T	GHD3R	GTT CCG TCT TCC AGT TCC TGT A	this study
Growth Hormone (GH) intron D	GHD3F	TCT CAT CAA GGT GAG TTT GAA CA	GHD3R	GTT CCG TCT TCC AGT TCC TGT A	this study
Growth Hormone (GH) intron D	GHD_int1F	CGA CAA GGA TTG TGG TTG TTG C	GHD_criR	GGC ATC TTC GTT TCT CTG GTT G	this study
Growth Hormone (GH) intron D	GHD3F	TCT CAT CAA GGT GAG TTT GAA CA	GHD_int1R	TCC CTT CAT GCA CAA AGG AGG T	this study
Growth Hormone (GH) intron D	GHD3F	TCT CAT CAA GGT GAG TTT GAA CA	GHD_CRI2R	GGA AGA GAA GGC CCC AAG AGT A	this study
Platelet-derived growth factor receptor *alpha* (PDGFRA)	PDGFRA Fa	GTC ATT GAG TCC ATC AGC CCT G	PDGFRA r	CAG TGG GTT TTA ACA TTT TCA CAG	this study
Platelet-derived growth factor receptor *alpha* (PDGFRA)	**PDGFRA 2f**	**AGC TGC CCT ATG ACT CCA GAT G**	**PDGFRA 2r**	**GCT CAA GCC ATA CGC TGT TCC T**	this study
Recombination activating gene 1 (Rag-1)	Rag1 250F	GAC ATG GAR GAC ATY ATY TTG	Rag1 1460R	ACT TAG ACT GCC TGG CAT TCA TTT	this study
Signal Recognition Particle 54 kD Protein (SRP54) intron C	SRP54C2F	GCG GAT GTG AAT ATT AAG CTT GT	SRP54C1R	GAC AAG CTC TTT GAA GAC AGC A	this study
Triosephosphate isomerase (Tpi) intron A	TpiA1F	AGT TCT TTG TCG GAG GCA AYT	TpiA1R	CAA AGT CGA TGT AGA TGG MWG GT	this study
Triosephosphate isomerase (Tpi) intron B	TpiB1F	GAG CCT TCA CTG GAG AGA TCA	TpiB1R	GAC TCT CCA AAG ACA TGC CTY CT	this study
Triosephosphate isomerase (Tpi) intron B	TpiB3F	CGC AGT TTT ACA AGC TTT GAT G	TpiB3R	CAA AGA CAT GCC TCC TCT CAG	this study
Triosephosphate isomerase (Tpi) intron B	TpiB3F	CGC AGT TTT ACA AGC TTT GAT G	TpiB4R	CTA TGG GAT AAA GCC TCA GGT G	this study
Triosephosphate isomerase (Tpi) intron B	TpiB3F	CGC AGT TTT ACA AGC TTT GAT G	TpiB5R	GTG CTA TGG GAT AAA GCC TCA G	this study
Vitelogenin	VTG F	ACC TCA ACT ACA TTC AGA CC	VTG R	GAG CTA TAT CCC AAG CAG G	C. Pinho (personal communication)
Anonymous markers					
CRI1	**CRI1 1F**	**ATC GCG ACT GGG AGT CTT ATT**	**CRI1 1R**	**ATG TTC TAT GCC CTC CCA GAG T**	this study
CRI2	CRI2 1F	GAA ATC TCT CTT CAG GGA AGC A	CRI2 1R	AAA CGG TTT GAA AGG AGT ACG A	this study
CRI2	CRI2 1F	GAA ATC TCT CTT CAG GGA AGC A	CRI2 2R	ATG AGC ATG AAG CAT TTG TCT C	this study
CRI3	CRI3 1F	CGA CTT TGA GAA AGC CTT TGA T	CRI3 1R	TCA ATT CTA TAA GCC GGG TCA G	this study
CRI3	CRI3 2F	ACT TGG TCC ACT CTG ACA CTC A	CRI3 2R	CCC AGT GGA TTG AGA GGT AGT T	this study
CRI4	**CRI4 1F**	**AGC TCT TTG AAG ACA GCA TTC C**	**CRI4 1R**	**CGC TTT GTG AAC TAC CAT ACC A**	this study
CRI4	**CRI4 1F**	**AGC TCT TTG AAG ACA GCA TTC C**	**CRI4 2R**	**CTC CAC ATC TGC TGA CAT GAT T**	this study
TVA4	TVA4 1F	ACA GTG CAA ATG CGT ACA ATT C	TVA4 1R	AGC AAG GAT CTG CTC AAG AAA C	Nadachowska & Babik (submitted)
TVA6	TVA6 1F	CTG CAT CAA ATG AGA GTC AAG C	TVA6 1R	ATC ATA TCC CCG ATT GGT GTA G	this study
TVA6	TVA6 1F	CTG CAT CAA ATG AGA GTC AAG C	TVA6 2R	GAT TGG TGT AGT CCC CAA GAA G	this study