Characterization of single nucleotide polymorphism markers for the green sea turtle (*Chelonia mydas*)

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Abstract

We present data on 29 new single nucleotide polymorphism assays for the green sea turtle, *Chelonia mydas*. DNA extracts from 39 green turtles were used for two methods of single nucleotide polymorphism discovery. The first approach employed an amplified fragment length polymorphism technique. The second technique screened a microsatellite library. Allele-specific amplification assays were developed for high-throughput single nucleotide polymorphism genotyping and tested on two Pacific *C. mydas* nesting populations. Observed heterozygosities ranged from 0 to 0.95 for a Hawaiian population and from 0 to 0.85 for a Galapagos population. Each of the populations had one locus out of Hardy–Weinberg equilibrium, SSCM2b and SSCM5 for Hawaii and Galapagos, respectively. No loci showed significant genotypic linkage disequilibrium across an expanded set of four Pacific nesting populations. However, two loci, SSCM4 and SSCM10b showed linkage disequilibrium across three populations indicating possible association.

Keywords: allele-specific amplification, Amplifluor, *Chelonia mydas*, green turtle, single nucleotide polymorphism, SNP

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Sea turtles are animals of considerable interest in conservation genetics due to their declining populations and complex life histories. Mitochondrial DNA has been extensively studied; however, further analyses involving nuclear DNA are necessary to resolve the complex population structure exhibited by sea turtles (Bowen & Karl 2007). An additional nuclear marker such as single nucleotide polymorphisms (SNPs) may enhance previous data and improve our understanding of marine turtle biology and evolution.

Two approaches were used to produce fragments of DNA for SNP detection in *Chelonia mydas*. The first approach employed amplified fragment length polymorphism (AFLP) techniques to generate and screen the sequences of random fragments of DNA as described in Roden *et al*. (2009). The second technique utilized a microsatellite library constructed by Genetic Identification Services (GIS) to identify clones with nonrepetitive *C. mydas* sequences.

Locus-specific primers were designed using Primer 3 software (Rozen & Skaletsky 2000) to amplify the largest product size possible for 16 candidate clone sequences from the library, and 13 sequences from the AFLP fragment isolation technique. Polymerase chain reaction (PCR) amplification included approximately 10 ng DNA, 0.2 mM each dNTP, 0.3 μM each primer, 1 U *Taq* Polymerase and 1× ThermoPol Buffer (New England Biolabs) in a 25-μL reaction volume. Cycling parameters included a 94 °C initial denaturation, followed by 30 cycles of 94 °C for 40 s, T_a for 40 s (Table 1), 72 °C for 40 s, and a final elongation step at 72 °C for 5 min.

After T_a optimization, loci were characterized by amplifying and sequencing a panel of *C. mydas* DNA samples. Sequencing reactions were performed with BigDye Terminator version 3.0 cycle sequencing kit (Applied Biosystems) and assessed with Sequencher 4.1.2 (Gene Codes Corp.). Any nucleotide difference at a single point along homologous sequences was recorded as an SNP.

SNP minor alleles in the screening panel ranged in frequency from 0.01 to 0.5; however, only alleles with frequencies > 0.04 were selected to be used for the study of population structure (Morin *et al*. 2008). For loci containing two or more SNP markers with minor allele frequencies (MAF) greater than 0.04, the two loci with the highest MAFs were selected to be converted into 2-SNP haplotypes to increase the power of population structure detection (Morin *et al*. 2008). Linked SNPs were not in phase, so that > 2 haplotypes were present for each locus in the population. Assays were designed for 32 SNPs and tested on two Pacific *C. mydas* nesting populations.

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Table 1 DNA fragments screened for SNP discovery using a panel of *Chelonia mydas* samples from Cyprus, Greece ($n = 4$), Taiwan ($n = 8$), Hawaii ($n = 9$), Quinatana Roo, Mexico ($n = 2$), Michoacan, Mexico ($n = 5$), Florida ($n = 3$), Puerto Rico ($n = 4$), and Galapagos, Ecuador ($n = 5$). All identified SNPs are indicated in the sequences submitted to GenBank. We calculated nucleotide polymorphism (θ) as described by Watterson (1975) as the number of polymorphic (segregating) nucleotide sites per nucleotide site using terminology in Nei (1987). When calculating total and overall values of θ, the average number of individuals was rounded to the nearest integer

Locus	Primer sequence (5′–3′)	T_a (°C)	Size (bp)	N	No. of SNPs	No. of SNPs MAF > 0.04	$\theta \times 10^3$	GenBank Accession no.
SNP D5	F: TCCACTGACTTGAGTTGTGTTTG	55	364	21	4	4	25.5	FI276984
	R: GCATAAAGCCTTCTCCACCT							
SNP D9	F: TAGCTGCCTAAACTATTTGGATGA	55	357	20	6	5	32.9	FI276985
	R: ACACAGGTGCCCAATTCAGT							
SNP C118	F: AGAATGGCTGAGTGATAAGCA	55	479	27	4	1	18.3	FI276983
	R: TCCATTATGAGAATCAACATGACA							
SNP B107	F: TTGAAAAATCAACAGTAACTATTCAGC	55	404	25	5	3	27.6	FI276975
	R: CAAATGGCAGAAAGGGAAAG							
SNP B109	F: CCGTCAGCAGGGTCACTTTA	55	412	20	7	6	39.9	FI276976
	R: GGAACCCATGTCCCTTACCT							
SNP C6	F: ATCCCTAGGCTTCTTCAACTAATG	55	351	25	0	0	0	FI276980
	R: CTTTTTGTATTGTACGGTTTCATCTT							
SNP B122	F: TCTTCCAAGCGGTTAAGCAT	55	418	28	4	1	20.8	FI276979
	R: TTTTGGATGAGAAGGAGAGGA							
SNP C12	F: CTGAATCAGAGACCATGCTGTT	55	207	35	11	7	110.3	FI276981
	R: CCATTATCCAAGGGACAATCC							
SNP B6ii	F: GGGATTGTGCCTTTTTGTTT	55	343	21	5	1	33.9	FI276974
	R: GCAGTGTGAGGTGTGATTGC							
SNP A6	F: TGGTGTCATGGACAATACCC	55	266	26	5	1	41.6	FI276972
	R: GTGAGAGCCAGGGTGTAAGG							
SNP B110	F: GGTGGGTATGGCATAGCAGT	55	371	22	3	1	18.6	FI276977
	R: CCAAGCTGAACAGTCCCAAT							
SNP C109	F: CCAAGCTTATCCCTAGGCTTCT	55	259	22	0	0	0	FI276982
	R: CTTCACCCTGGAGTCCGTAA							
SNP A7	F: CTTGCGTGGAAAAACCAAGT	55	349	20	5	3	33.7	FI276973
	R: ACTCTGAAACAACCCCCTCA							
SNP C117	F: AGTGCAACAGCCCCTCAC	55	271	23	8	4	67.2	FI276971
	R: TTTTCCCTATTGATTTTCTGTGAA							
SNP B116	F: ACTCATGTTTCATGGCCTCA	No amp	No amp	NA	NA	NA	NA	FI276978
	R: TTGAAAGAGGCTTACCTTAGAGACA							
SNPD103	F: TTTCACATACATTGGGATGGT	55	290	23	1	0	7.8	FI276986
	R: CCACCCATAATTGTCACAAGC							
Subtotal			5141	358	68	37	29.8	
Frag18	F: GCCCACTGTGTTTAGTTGACC	55	139	37	3	2	44.3	FI276994
	R: TGTTTATGTATCTCTAAAACCTTGGAA							
Frag5	F: ACCATGTCCCACCCACTG	55	179	32	2	2	23.6	FI276989
	R: GGAAACCTACTAAAACTGCTCCA							
Frag14	F: CCGAGTTGCAGACTGTAGCA	55	208	39	1	1	9.8	FI276992
	R: GAACGTTTGGATCATGGTATTT							
Frag21	F: ACCATGTGCCAAATCGTGAG	55	138	38	4	3	44.4	FI276995
	R: CAAACTGAGAGAGCCCCAGA							
Frag1	F: GGTACCATGGGCATCATAGG	55	178	29	0	0	0	FI276987
	R: GGGTGAAACATGGGAGCTAA							
Frag10	F: TCTTCCACTGTGCCTTTTCC	55	170	37	3	1	36.2	FI276990
	R: TTTGCCTGTGCAGTACTATTACAA							
Frag11	F: GCCCCTTGAACAGAAGTGAG	55	314	22	9	5	65.9	FI276991
	R: CTCCCAAGCTTTCCTCTCCT							
Frag4	F: CATCGTAGGGACCCATGAAA	55	483	27	5	3	22.7	FI276988
	R: AGGAAGTCCAGATGCCACAT							
Frag16	F: TGCTCACTACAGCCTTCCAG	57	173	36	3	2	35.8	FI276993
	R: TTCTGACGTTCCACAGGAGA							

Table 1 *Continued*

Locus	Primer sequence (5′–3′)	T_a (°C)	Size (bp)	*N*	No. of SNPs	No. of SNPs MAF > 0.04	$\theta \times 10^3$	GenBank Accession no.
Frag44	F: AAGGCAGGGTCAGAATTGAG R: TCAGTGCTTATTGCTCCCTCT	55	69	23	0	0	0	FI276999
Frag40	F: AGATCAGGCCCTAAATGTCG R: TTGGAGTTCCAAACCACAGA	60	221	35	3	2	28.2	FI276997
Frag42	F: ATTTGAAGACAAGTAAATGTTCCAT R: TTTCTCCATAGCTCTTAGGCAAA	55	129	39	2	1	31.5	FI276998
Frag30	F: AGGAAGTTGGGTGCGTTATG R: CAATGACATCAACATACATGTCTCC	58	146	23	0	0	0	FI276996
Subtotal			2547	417	35	22	29.1	
Overall			7688	775	103	59	29.3	

N, number of individuals sequenced.

Allele-specific amplification assays were designed to be genotyped with the Amplifluor SNPs HT Genotyping System (Millipore Corporation). This is a single tube system utilizing five primers designed with Amplifluor Assay-Architect Software. The detection system includes two allele-specific primers containing 5′-Amplifluor-complementary oligonucleotide tags, a common reverse primer, and two universal Amplifluor SNP primers (Myakishev *et al*. 2001; Giancola *et al*. 2006). Fluorescence was detected in real time using a Stratagene Mx3000P QPCR System.

Multiplex sets were designed to amplify all SNP regions, generating pre-amplified product to be used as the template for Amplifluor reactions (Morin *et al*. 2007). Multiplex primers included a reverse primer designed by Assay-Architect software and a complementary flanking primer designed using Primer 3 for each SNP genotyping locus. The flanking primer was designed to generate a product 5–30 bp larger than the Amplifluor product. Multiplex PCR conditions were performed according to Rompler *et al*. (2006). All 32 assays were performed in a 20-μL total reaction volume with 1× PCR buffer (1.5 mM $MgCl_2$) (QIAGEN), 2.5 mM $MgCl_2$ (for a total of 4 mM $MgCl_2$), 1 mg/mL BSA, 0.25 mM each dNTP, 1 U HotStar*Taq* DNA polymerase (QIAGEN), approximately 1 ng DNA, and 0.15 μM each primer in the multiplex primer set. Multiplex cycling conditions included 95 °C for 10 min, followed by 30 cycles of 95 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 4 min.

The pre-amplified multiplex product was then diluted 1:1000. Four microlitres of each diluted product was added to 96-well PCR plates, dried at 56 °C, sealed and stored at room temperature. Amplifluor SNPs HT Genotyping System was used for all assays, varying only the annealing temperatures, magnesium chloride concentration of the Amplifluor 10× buffer (Millipore Corporation), or the primer concentrations in the 20× SNP specific primer mix (Table 2). In addition, the 20× Amplifluor SNP FAM and JOE primers were each used at a concentration of 0.15 μM instead of the recommended 0.25 μM per reaction.

Of 32 assays tested, 29 were optimized and successfully produced genotypes. An overall average error rate of genotype data compared to sequenced control samples was calculated to be 0.0056 from five confirmed errors out of 893 genotyped and sequenced samples from the SNP discovery panel. No markers showed significant genotypic linkage disequilibrium across an expanded set of four Pacific nesting populations. However, SNP markers SSCM4 and SSCM10b showed linkage disequilibrium across three populations indicating possible association.

The presented data reveal that green turtles have a high frequency of SNPs, and suggest the potential for an abundance of variable loci within the genome. These SNP markers are the first to be designed for genotyping green sea turtle populations and will provide a necessary addition to the genetic tools employed for understanding population structure and developing regional conservation management strategies for this threatened/endangered species.

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Table 2 SNP genotyping and multiplex PCR primers tested on *Chelonia mydas* nesting populations from Galapagos, Ecuador (Gal; $n = 56$) and Hawaii, USA (HI; $n = 126$). Note (a) the polymorphic nucleotide is not always at the most 3′ site, (b) sometimes primers were designed for the complementary strand, (c) loci with 'b' at end of name indicate SNP with second highest MAF to use with associated primary marker as phased haplotypes. MAFs are reported as both populations combined

SNP Locus	SNP location	SNP type	MAF	Flanking primer	Allele-specific primers	Reverse primer	Amplifluor buffer ($MgCl_2$) mM	T_a (°C)	H_O (Gal/HI)	H_E (Gal/HI)
SSCM1	59	C/G	0.46	CATCACTAGAAGCTAACCAGGAAA	†GGAAAGCTGGGGAAATTCTTTAGTG *GGAAAGCTGGGGAAATTCTTTAGTC	GGTTTGTCTGGTTTATACCAAAGTT	1.8	58	0.32/0.46	0.30/0.48
SSCM2	261	A/C	0.38	GGAATAAAAAGAATCCTAACAATGG	*TCCTAACAATGGTATTGGTCCATTACTAG †TCCTAACAATGGTATTGGTCCATTACTAT	CCCCCAAGTGCAGAACA	1.8	66	0.60/0.48	0.50/0.45
SSCM2b	223	A/G	0.33	TTCCTCCCACCCCCAAGT	†GAACAKAATAGTTATTGAACACTTCTGCA *GAACAKAATAGTTATTGAACACTTCTGCG	CCTAACAATGGTATTGGTCCAT	1.5	55	0.55/0.29	0.46/**0.44**
SSCM3‡	57	A/C	0.27	TCCACTGACTTGAGTTGTGTTTG	*GCATTTTTACCAGTCTAAATACAGCTTTCTG †TCTGCATTTTTACCAGTCTAAATACAGCTTTCTT	TCCTCTGATGACATTATTATCTCCAT	1.0	60	0.85/0.37	0.50/0.31
SSCM3b	262	A/G	0.34	CAGCCTCAGCTCTGGATCTC	*TTCTCTATTCCGTGCTGCCAG †TTCTCTATTCCGTGCTGCCAA	CCACCCTATCAGTTTCCTCAAT	1.8	58	0.07/0.95	0.07/0.50
SSCM4	308	A/G	0.36	CCCAGCTCCAATTCTACTCC	†TATCAGACACAGATTATGGTCAACTAT *CAGACACAGATTATGGTCAACTAC	CTGGTTTGAACATCCAGTTTCAT	1.8	60	0.38/0.52	0.37/0.49
SSCM5	356	A/C	0.20	TGCTTTACAAAAACTGAAGCACA	*GAAAGTCACAACCTGCACAAGA †AAGTCACAACCTGCACAAGC	GCCCAATTCAGTTAGGTTCCTT	1.8	58	0.25/0.28	**0.40**/0.28
SSCM5b	244	C/G	0.18	TGGTCAGCAGATAAAATAACCA	†TGAGTTTTCCACTTACCAGGAATTACTTTTAAC *GAGTTTTCCACTTACCAGGAATTACTTTTAAG	GACAGTGATAATGCAGGTGTCAA	1.5	55	0.27/0.27	0.35/0.27
SSCM6	87	G/T	0.45	ATCAGCCCCTGAAGTCCTTT	†ATGTCACGGTAGAGCTCATTCC *GATGTCACGGTAGAGCTCATTCA	CTGCTATGGGGATAAACAGAGTT	1.5	58	0.55/0.51	0.50/0.50
SSCM6b	123	G/T	NA	CAACAGCCAAAGTGACCATT	†AGCCCCTGAAGTCCTTTTCCA *GCCCCTGAAGTCCTTTTCCC	TACCGTGACATCTGGTGGTGA	NA	NA		
SSCM7	153	G/T	NA	TGCCATTACAGATGGCAAT	†GGCAAATCTGTTGCATTATGGTACG *ATGGCAAATCTGTTGCATTATGGTACT	GACACCATTGGGTATACTCAAGT	NA	NA		
SSCM8§	329	C/T	0.08	TTTCCACCATTTTTATCTCCA	†TGGGGAGAGCTGTGCTTAGC *GTGGGGAGAGCTGTGCTTAGT	ACCCCCTCATAACACATAGGAT	1.5	58	0.44/0.04	0.35/0.04
SSCM8b	193	C/T	0.09	TGGAGATAAAAATGGTGGAAA	*TTTGAAGCCGTGCCTCG †TCTTTTGAAGCCGTGCCTCA	CCCCCATAGCCTCAACTCTTT	1.0	55	0.38/0.01	0.40/0.01
SSCM9	77	A/G	0.47	GGTGGGTATGGCATAGCAGT	*TGAAACATCAGGAGACTTAACCAACTA †AACATCAGGAGACTTAACCAACTG	GAGATTGCTCTGTGCAGTCAAA	1.5	58	0.45/0.49	0.50/0.50
SSCM10	184	C/T	0.14	GTGTCGGGGCTGAAGTGG	†ACGGARTTATTTTTTTAACAATACGCAT *ACGGARTTATTTTTTTAACAATACGCAC	GAGGATAACAGAGATAAAACACACGTTTA	1.5	58	0.02/0.28	0.02/0.32
SSCM10b	162	A/G	0.48	GTGTCGGGGCTGAAGTGG	†GTGGGCACACMTACGGAG *TGTGGGCACACMTACGGAA	GGATAACAGAGATAAAACACACGTT	1.5	55	0.63/0.43	0.50/0.50
SSCM12	157	C/T	0.10	TTTCATCCGAGGAGAGAACG	*AGAACGCCCTCTTCAAAGCTAC †GAGAACGCCCTCTTCAAAGCTAT	GCTCCAGTTTGCTTGCAAAAT	1.5	55	0.33/0.16	0.28/0.15

Table 2 *Continued*

SNP Locus	SNP location	SNP type	MAF	Flanking primer	Allele-specific primers	Reverse primer	Amplifluor buffer ($MgCl_2$) mM	T_a (°C)	H_O (Gal/HI)	H_E (Gal/HI)
SSCM13	149	A/T	0.40	CAAACTGAGAGAGCCCCAGA	†GCCCCAGAKCCCCACAAA *GCCCCAGAKCCCCACAAT	AAACTGACAAAATCCCCAGGTG	1.8	58	0.41/0.45	0.50/0.45
SSCM13b	107	A/G	0.03	CTTTGGGAATAATGGGGAAG	*AACCTCTGGATTTGACCTGCG †GAACCTCTGGATTTGACCTGCA	ACCTGGGGATTTTGTCAGTTT	1.5	55	0.09/0.06	0.09/0.05
SSCM14	53	A/T	NA	TGACTTAATCACATTTATACTGTGCAA	†ATCACATTTATACTGTGCAAGCAGAATT *ATCACATTTATACTGTGCAAGCAGAATA	CTTTCATCGTAGGGACCCAT	NA	NA		
SSCM14b	188	A/G	0.01	TGGATGAGTACATTCTTTGTCATTT	†GATGACAATTTCCTAACTCAAAAAGTGTTGT *GATGACAATTTCCTAACTCAAAAAGTGTTGC	GTTCTGTGATCAGTTCCTCTTT	1.5	60	0.09/0.00	0.09/0.00
SSCM15	141	C/T	0.09	AGCAGGAAGCCAGTGCTTAG	*CGTAAGGGGCATGTTTTCTTCAAAGAG †CGTAAGGGGCATGTTTTCTTCAAAGAA	TAAGGAGGAGCCGGCATTCA	1.0	53	0.27/0.13	0.23/0.12
SSCM15b	167	A/G	0.06	CACCCCAACAGGTCCCTAA	*TTGAAGAAAACATGCCCCTTACA †GAAGAAAACATGCCCCTTACG	AGCAGGAAGCCAGTGCTTA	1.5	55	0.00/0.15	0.00/0.16
SSCM16	152	C/T	0.05	AAACAAAGCAGCAATGCACA	†TGAGGAAACTGAGAGCTGAAGC *CATGAGGAAACTGAGAGCTGAAGT	CTTTCTCCATAGCTCTTAGGCAAA	1.5	56	0.00/0.14	0.00/0.13
SSCM17	94	C/T	0.04	ATTCAAACCAGAGCGAGCAT	†ACAGAAAGGAAACCTCTCCAAAC *CACAGAAAGGAAACCTCTCCAAAT	GAGCAGAGTGGGTGTGAAT	1.5	60	0.00/0.10	0.00/0.10
SSCM18	235	A/C	0.05	CCGTGTGAAGCGTGACTG	†GCAGGGTTAAAAATAGCCATGTAA *TGCAGGGTTAAAAATAGCCATGTAC	AAGTAGATGTCCAGGGTCCCCA	1.0	55	0.29/0.00	0.27/0.00
SSCM19	206	C/G	0.13	ATGAAGCATGCGTTCTTGTG	*AATGCTGGGGCCAGAATCTTTG †TGCTGGGGCCAGAATCTTTC	CGTTTGGATCATGGTATTTATCAGAACA	1.5	55	0.02/0.29	0.02/0.29
SSCM20	32	C/T	0.42	TGGTTTGCAGAGATGCTGAG	†GAGATGCAAAAACCCAGCACCA *AGATGCAAAAACCCAGCACCG	CCTTGAACAGAAGTGAGCTGA	1.0	55	0.16/0.48	0.15/0.49
SSCM20b	324	A/G	0.15	AAGAAGGGATTCCGGAGAAG	*TCGTATGTTGTAACTAGTGCTGYG †CGTATGTTGTAACTAGTGCTGYAA	GCTTTCCTCTCCTTACCAGTGA	1.5	58	0.43/0.11	0.45/0.12
SSCM21	87	A/G	0.17	GGCCCTAAATGTCGTCAAAA	†GTGGTAGCCCATCATGTGTG *GGTGGTAGCCCATCATGTGTA	CAGTGTTTTGTCCAGTCTGGAT	1.0	57	0.18/0.28	0.22/0.31
SSCM21b	151	A/G	0.07	GGTGGTAGCCCATCATGTGT	†CTGGAGTGAGGGATGGACTAG *GGAGTGAGGGATGGACTAA	TTCAGTGGCTCTTCCTACCT	1.0	57	0.27/0.06	0.26/0.05
SSCM22b	44	A/G	0.18	GAATGGAACTATGCCCGTTT	†GGGGATACATTGCACACGTTTT *GGGGATACATTGCACACGTTTC	CCACTGTGTTTAGTTGACCCAT	1.5	55	0.04/0.36	0.08/0.37

H_O, observed heterozygosity; H_E, expected heterozygosity, with significant deviations from Hardy–Weinberg equilibrium shown in bold; *FAM tail (GAAGGTGACCAAGTTCATGCT); †JOE tail (GAAGGTCGGAGTCAACGGATT); ‡adjusted 20× SNP specific primer mix (0.25 μM JOE allele-specific primer); §adjusted 20× SNP specific primer mix (0.125 μM FAM allele-specific primer).

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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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