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Development of thirty-four new microsatellite loci and multiplexing of seven existing loci for *Zootoca vivipara* (Squamata: Lacertidae)

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Abstract

Development of thirty-four new microsatellite loci and multiplexing of seven existing loci for *Zootoca vivipara* (Squamata: Lacertidae). Few microsatellite loci exist for the European common lizard, *Zootoca vivipara,* a common model species in studies of population dynamics, sexual selection, population genetics, parity evolution, and physiology. The existing primers did not amplify in all lineages, and multiplexes were not optimized. A total of 34 new polymorphic microsatellite markers have been developed for this species and tested in 64 specimens belonging to oviparous and viviparous clades (B and D). The microsatellites were combined into seven different multiplexes. Results showed that all but one loci successfully amplified in all samples and both clades. The number of alleles detected per locus ranged 7–22 alleles and the effective number 1.58–7.82. The observed heterozygosity ranged 0.312–0.930, showing that all loci were highly variable. Oviparous and viviparous clades exhibited significant genetic differences (in F_{ST}). In addition to these new markers, the seven previously published and widely used microsatellite loci have been multiplexed and tested in oviparous clades. These innovations will allow for timesaving and robust analyses in *Zootoca vivipara,* boosting evolutionary and population studies and easing paternity analyses.

Keywords: European Common Lizard, *Lacerta vivipara*, multiplex, NGS, nuclear DNA, viviparity, oviparity.

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Resumo

Desenvolvimento de trinta e quatro novos locos de microssatélites e otimização de PCR multiplex de sete locos existentes para *Zootoca vivipara* (Squamata: Lacertidae). Existem poucos locos de microssatélites para o lagarto-comum-europeu, *Zootoca vivipara,* uma espécie-modelo comum em estudos de dinâmica de populações, seleção sexual, genética de populações, evolução do modo reprodutivo e fisiologia. Os oligonucleotídeos existentes não amplificaram em todas as linhagens, e a reação em cadeia da polimerase (PCR) multiplex não foi otimizada. Um total de 34 novos marcadores polimórficos de microssatélite foi desenvolvido para essa espécie e testado em 64 espécimes dos clados ovíparo e vivíparo (B e D). Os microssatélites foram combinados em sete diferentes agrupamentos . Os resultados mostraram que todos os locos, com uma única exceção, amplificaram com sucesso em todas as amostras e em ambos os clados. O número de alelos detectado por loco variou entre 7 e 22 e o número efetivo, entre 1,58 e 7,82. A heterozigosidade observada variou de 0,312 a 0,930, mostrando que todos os locos foram altamente variáveis. Os clados ovíparo e vivíparo exibiram diferenças genéticas significativas (em F_{ST}). Além desses novos marcadores, os sete locos de microssatélite previamente publicados e largamente utilizados foram otimizados em PCR multiplex e testados em clados ovíparos. Essas inovações permitirão análises rápidas e robustas em *Zootoca vivipara,* impulsionando estudos evolutivos e populacionais e facilitando análises de paternidade.

Palavras-chave: DNA nuclear, *Lacerta vivipara*, lagarto-comum-europeu, PCR multiplex, NGS, oviparidade, viviparidade.

Introduction

The European Common Lizard, *Zootoca vivipara* (Lichtenstein, 1823), is the most widely distributed reptile species of the world (Guillaume *et al.* 2006). Its distribution ranges from Ireland and western Spain in the west to Japan (Hokaido) in the east, and from the Balkans and Pyrenees in the south to the polar circle in the north; there are several clearly distinct lineages across Eurasia (Clades A–F; Surget-Groba *et al.* 2006). One of these clades has been proposed to be a new species (Clade A; Cornetti *et al.* 2015a). The species is reproductively bimodal; two lineages are oviparous (Clades A, B) and all the other clades are viviparous (Clades C–F). Owing to the range and reproductive bimodality, *Z. vivipara* is a highly interesting species in terms of its evolution, geography, and population dynamics. Evolutionary studies (e.g., Surget-Groba *et al.* 2006, Cornetti *et al.* 2015b) and studies analyzing individual paternities (e.g., Laloi *et al.* 2004, Fitze *et al.* 2005, 2008, Richard *et al.* 2005, 2009, Eizaguirre *et al.* 2007, Fitze and Le Galliard 2008, Le Galiard *et al.* 2008, San-Jose *et al.* 2014, Breedveld and Fitze 2016) have been conducted on this species, whereas population genetic studies are rather rare. Such studies need reliable, time- and cost-efficient genetic tools; however, few microsatellite loci had been developed, protocols did not work in all lineages (Boudjemadi *et al*. 1999, Remon *et al.* 2008, Molecular Ecology Resources Primer Development Consortium 2011), and multiplexes were not optimized (Laloi *et al.* 2004). Efficient multiplexing (co-amplification of several loci in a single PCR) can significantly decrease the cost of genotyping and increase throughput of microsatellite loci (Guichoux *et al.* 2011).

We have used next generation sequencing methods to develop a large new panel of microsatellite loci and generated cost-effective multiplexes for new and existing microsatellite loci.

Materials and Methods

For the development of new microsatellite loci, a total of 64 *Zootoca vivipara* individuals (Table 1) were sampled. Thirty individuals

belonged to Clade D (the Eastern Viviparous Clade) and another 34 belonged to Clade B (the Western Oviparous Clade; Surget-Groba *et al.* 2001). Their genomic DNA was extracted from ethanol-preserved lizard tissue using DNeasy Blood & Tissue Kit (Qiagen, Verlo, Netherlands), which produces DNA of better quality than other methods (Horreo *et al.* 2015).

The genomic DNA of one specimen of the Western Oviparous Clade was enriched following the protocol of Santana *et al.* (2009). A 454 library was obtained from a partial run using the 454 Life Sciences/Roche GS-FLX genome sequence system (Roche Applied Science) (Margulies *et al.* 2005). A total of 709,643 sequence reads (153,531,887 base pairs) was generated, of which 38,000 contained a minimum of five tandem repeats: 17,297 dinucleotides; 16,432 trinucleotides; 3,750 tetranucleotides; 337 pentanucleotides; and 184 hexanucleotides. Ninety-eight of these sequences (24, 58, and 16 tri-, tetra-, and penta-nucleotides, respectively) were selected to design primers using Primer3 (Rozen and Skaletsky 2000). Selected repeats were amplified in eight specimens belonging to all clades described (one individual per lineage: A from Italy, B1 from Spain, B2 from France, C and E from Austria, D from Romania, F from Hungary, and G from Galicia Spain) (Surget-Groba *et al.* 2001, Milá *et al.* 2013), using 10–100 ng of DNA in a total reaction volume of 10 µL and the Taq DNA Polymerase (5PRIME GmbH, Germany). The proportions of the primers, Taq, 5Prime mastermix, magnesium, and molecular-biology grade-water followed the manufacturer's protocol. The PCR started with an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 57°C for 20 s, extension at 70°C for 30 s, and a final extension of 72vC for 10 min. PCR products were visualized in 2% agarose gels. If the amplification was successful in all eight specimens, the Forward primer was labelled with a fluorescent-dye (VIC, NED, PET and FAM). Thereafter, successful loci were amplified in a subset consisting of 16 samples of the 64 studied individuals (B and D; Table 1). PCR conditions corresponded to those described above and the total reaction volume was 25 µL. PCR products were visualized using an automatic sequencer (an ABI 3100, Applied Biosystem) and the software GeneMapper 4.0 (Applied Biosystems). Thirty-four loci (6, 19 and 9 tri-, tetra-, and penta-nucleotides, respectively; Table 2) were polymorphic and exhibited consistently good electropherograms.

The 34 microsatellite loci were combined in seven multiplexes (Table 2), and their amplification followed the "QUIAGEN Type-it Microsatellite PCR Kit" protocol and an annealing temperature of 57°C. In these multiplexes, 31 primers were employed using the manufacturer's protocol and the proportions of ZV12 (Multiplex 3), ZV29 (Multiplex 6), and ZV32 (Multiplex 7) were 1.5 times higher than those of the other primers. Multiplexes were then amplified in the 64 samples and Tandem v.1.09 (Matschiner and Salzburger 2009) was used for allele binning. Thirty-three loci successfully amplified in all 64 samples. Locus ZV14 amplified in all oviparous samples (Clade B), but it did not amplify in most of the 30 viviparous specimens (Clade D; Table 1) despite its amplification during the development of the method.

Table 1. Sampling details for *Zootoca vivipara*. The clades have been named according to Surget-Groba *et al.* (2001). *N* = sample size. Acronyms: ND = newly developed microsatellites; EM = existing microsatellites.

Microsatellites	*N*	Clade	Country	Reproductive mode
ND	30	D	Hungary/Romania	Viviparous
ND	34	B	France/Spain	Oviparous
EM	12	B	Spain	Oviparous

Table 2. Genetic variability, primers, and repeat motif of the 34 newly developed and the seven previously known (Boudjemadi *et al*. 1999) microsatellite loci of *Zootoca vivipara* (ZV1–ZV34 and LV 1–4, respectively). The genetic variability of the newly developed loci was calculated based on 64 specimens and the 7 previously existing (named "Lv") on another 12 specimens. Acronyms: Na = number of alleles per locus; Eff_Na = effective number of alleles per locus; H_E = expected heterozygosity; H_O = observed heterozygosity; Motif = repetition motif; Color = used fluorescent dye; Mn = multiplex number.

Locus	Size (bp)	Eff_Na	Na	Ho	He	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Motif	Color	Mn
ZV1	141–225	7.82	20	0.732	0.904	TGACTCCACTTTGCTTGCAT	CACATGTGAAGCTGCATCTAAG	$(ATCT)_{12}$	NED	1
ZV2	191–227	5.22	10	0.501	0.840	GAGCACCTCCAAATTTTTACTGTT	GTTTGTTGCAACCCACACTG	$(GATA)_{11}$	FAM	1
ZV3	100–176	5.61	19	0.765	0.846	TGCCTTGAATCCCAGTTCTC	TGAGAAGATACCCGGTCAGG	$(GATA)_{10}$	FAM	1
ZV4	117–157	5.19	11	0.788	0.830	CACTTTGACGGGTTTGGACT	CCATCCCTGTAGAAAAGGCA	$(AACA)_{11}$	VIC	1
ZV5	185–248	5.07	12	0.559	0.833	AAGTTGCAGGGAACTAGGCA	TGTTGCCATCTATTTGTCTCAA	$(TTA)_{16}$	VIC	1
ZV6	179–239	5.27	13	0.724	0.835	ACCAACAACCACCTCAACCT	TCTGGACATATAGCCTGGTCC	$(TATTC)_{11}$	FAM	2
ZV7	240–348	5.30	18	0.694	0.837	CAGAGCTGAGCCTGGAAGTT	AGCACCCGAGAGTGAAAAAC	$(AGAT)_{10}$	FAM	2
ZV8	215–287	5.49	14	0.811	0.841	CTCCAATTTGGCAGGTGAAT	TCTTCCTTGCTGCCAAGACT	$(GTTT)_{11}$	NED	2
ZV9	203–242	6.01	12	0.747	0.859	AGGTGTAGAGAATGGGCACG	ATGGCAGGTATCTGGAGCAT	$(TAG)_{13}$	PET	2
ZV10	119–224	6.21	17	0.705	0.869	AACCATCTTGGGTGTCTTGG	TCAGGAATCTTTGGATGGAGA	$(ATAAC)_{19}$	VIC	2
ZV11	220–300	4.90	16	0.774	0.819	CAAGAGTCTCCTCCAGCACC	TTGGTCCATGCATGAAAATG	$(GATA)_{11}$	PET	3
ZV12	121–205	2.64	13	0.609	0.639	TGTTTGTTTAATCCCCCGTC	GGAGAAGCAATGGAAACTGG	$(TTGT)_{9}$	PET	3
ZV13	203–245	3.80	14	0.577	0.762	TGAGCCACAGTCATCAAAGG	TGCAACACCTAGAGGTCTTCTG	$(TAT)_{16}$	FAM	3
ZV14	140–164	3.00	7	0.710	0.796	CCCACCCTTCACCATAATGT	CCAGATGAGCGGGGTATAAA	$(AAC)_{11}$	FAM	3
ZV15	110–210	6.67	20	0.695	0.880	TGTACTGATATGATGCAAAACACC	CATTAGGCGGCAGATTCATT	$(AGTAT)_{16}$	PET	4
ZV16	177–241	3.12	11	0.893	0.692	TGTAATCTGATCCGCTGCTG	CTGAAGGCAGCCCTGTTTAG	(ACAT)9	FAM	4
ZV17	171–236	3.85	14	0.649	0.766	TTGAATGCTTCCTCCCTCTG	TTCGAAAAGCAGGAATTGAA	$(ATGTA)_{10}$	NED	4
ZV18	141–206	5.67	14	0.816	0.846	CAGTATAGGCTTGGGGTCCA	TCTTCAGGCCTCGTTTCACT	$(TTTCT)_{9}$	VIC	4
ZV19	153–201	6.75	11	0.836	0.876	TGCAGGTGTACACTGGGCTA	TGGGCTAAAGCCACTAGAGC	$(TGTA)_{9}$	NED	5

Table 2. *Continued.*

Locus	Size (bp)	Eff_Na	Na	Ho	He	Forward Primer (5′-3′)	Reverse Primer (5′-3′)	Motif	Color	Mn
ZV20	126–174	5.64	12	0.579	0.852	AGGGTGTTTCTGTGAGTCGG	CCTCTCCCCTCTTTTTCCAG	$(AAGA)_9$	PET	5
ZV21	095–139	5.10	10	0.649	0.832	CTTGCAACACCAGGAACTCA	TGGTTTAGACCACAGCACCA	$(GATA)_9$	FAM	5
ZV22	152-204	1.58	9	0.312	0.383	GGCAAGGTAACAATTGGCAT	TTGCCTACAGCAAATGGATG	$(GATA)_{12}$	FAM	5
ZV23	212-256	3.00	12	0.619	0.686	GGAGGCTACTATTGGGGCTC	AGCATTCATTGTGAGTTATGGC	$(GATA)_{12}$	FAM	5
ZV24	164–224	3.92	10	0.727	0.766	GCCATGAAAGTCGTGTTGTG	TGTAAACGGTCCCACGAACT	$(GTTT)_{10}$	VIC	5
ZV25	081–126	4.14	9	0.777	0.779	AGAGCATGAGGTCAGAGGGA	CCCCCACCCACATATTACAG	$(TTCTA)_{10}$	FAM	6
ZV26	213–283	4.99	14	0.683	0.825	GCCAAGCAAATTTCAAGTATGT	GAGCTCACTCCATTGTGCAG	$(TTCTA)_{10}$	FAM	6
ZV27	104–149	3.49	10	0.689	0.733	GATGGCAAGTGTGGCAGTAA	TGCTTGAAATGAGGTGTGGA	$(TATAG)_{10}$	VIC	6
ZV28	154–198	5.18	13	0.746	0.831	CATGGTTCCAACAATCCCTT	CTGCTGCTTGTGGAACTGAA	$(TTA)_{16}$	FAM	6
ZV29	085–165	3.10	11	0.418	0.704	TCACATGAGTCAACGGCCT	GTGAGTCATCTGCGACTGGA	$(TGTTT)_9$	PET	6
ZV30	113–189	5.81	13	0.832	0.854	CCCGGGAGTAAGAGGAGGTA	GTGCAAGTGGGTTGATGTTG	$(TCTT)_{10}$	NED	7
ZV31	111–151	6.12	11	0.506	0.870	CAGAAGAATGCCACTCTGGA	AGGTCTCTTGCCCACTTTGA	$(TTTC)_{10}$	PET	7
ZV32	202–298	6.45	20	0.930	0.869	CAGGTTAAGAACGGATCTCCA	AGCCTGCACATCCCAGTATC	$(GATA)_{10}$	FAM	7
ZV33	105–137	4.01	8	0.708	0.773	GACACCCTTGTTGCCTCATT	TCCCTCCCTGTCTGAAAGAA	$(TGTT)_9$	FAM	7
ZV34	127–208	6.04	22	0.906	0.856	GGAGATTGTTAGCCGCTTTG	CAGCAATCTAGTCTGCTTCCA	$(TCT)_{22}$	VIC	7
Lv-4-72	122–146	7.78	7	0.917	0.909	TGCCGTCAAAGCCAAACAAG	CCGCCCTCCACAATACACCT	$(AC)_{18}$	NED	8
Lv-2-145	296–310	3.39	4	0.667	0.739	CCATTGTAGGCTCAGGTTG	GGTGCCAACTATGCAGG	$(TG)_{20}$	NED	8
Lv-4-x	167–190	6.86	9	0.917	0.890	TGGATTAGAGGCTGAAAGAG	TGAGAAGGCTGTGAATGTG	$(GT)_{22}$	NED	8
Lv-4-∝	157–181	5.33	7	1.000	0.841	ATTTACCTGCAGGGAACAGA	CCAGAAAGCATTTCCACAC	$(AC)_{14}(GA)_{11}$ $(CAGAGA)_9$ $CAGAT(AG)_3$	FAM	8
Lv-1-139	134–140	1.97	3	0.333	0.527	GGGAGATGTTGCCTTATGG	CTGCATTTAAAACTGAAGTGGC	$(GA)_{26}$	FAM	8
Lv-4-115	125–158	5.14	8	0.917	0.837	CCCAACCCACAAGACTGA	CCGGTGTACTCAATGATGCT	$(CA)_{17}$	PET	8
Lv-3-19	134–162	9.29	12	1.000	0.928	GCTGTTGCTATTTTGTATGCTTA	CCCTGTGACTGTCCTCAGAG	$(AC)_{22}$	VIC	8

In addition to all this, seven previously published (Lv-3-19, Lv-4-72, Lv-4-alpha, Lv-2-145, Lv-4-X, Lv-4-115, Lv-1-139; Table 2) (Boudjemadi *et al.* 1999) and commonly used microsatellite loci (e.g., Breedveld and Fitze 2016) were multiplexed to save money and time. PCR conditions were the same as those described above, except that annealing was conducted at 58°C. In the newly developed multiplex, the primers Lv-4-X, Lv-4-alpha, and Lv-3-19 were employed in a proportion three times higher than the rest. A set of 12 oviparous samples was amplified using the multiplex protocol and the previous protocols (following Laloi *et al.* 2004).

The genetic variability of all the loci (number of alleles per locus, effective number of alleles per locus, and observed and expected heterozygosity), as well as the genetic differentiation among sample groups (F_{ST}) were calculated with GenoDive 2.0b25 (Meirmans and Van Tienderen 2004). The linkage disequilibrium among pairs of loci was calculated with GENEPOP v.4 (Rousset 2008).

Results

The 34 newly developed loci exhibited high genetic variability (Table 2). The mean number of alleles per locus (± standard deviation, obtained through jackknifing) was 13.29 ± 0.63; the effective mean number of alleles per locus was 4.89 ± 0.24. The mean expected heterozygosity was 0.79 ± 0.02 and the mean observed heterozygosity was 0.69 ± 0.02. No linkage disequilibrium was detected among pairs of loci. The fixation index (F_{ST}) among clades (B and D) using 33 loci was significant (p = 0.01, F_{ST} = 0.082), indicating that genetic differences among clades can be detected with the newly developed loci.

In the case of the seven previously published loci, both the multiplex protocol developed here and the previous protocols rendered the exactly same genotypes. The mean number of alleles per locus (± standard deviation) was 7.57 ± 1.21; the effective mean number of alleles per locus was 5.68 ± 0.95. The mean expected heterozygosity was 0.81 ± 0.05 and the mean observed heterozygosity was 0.82 ± 0.05. Thus, the range of variability of both the newly developed and the old loci was similar (A_e new: 1.58–7.82; A_e old: 1.97–9.29). ZV22 was the least variable of the new loci and, of the previously published loci, LV-1-139 was the least variable in the oviparous clade, but not in the viviparous Clade E (Boudjemadi *et al.* 1999).

Discussion

Thirty-four newly developed, highly polymorphic microsatellite loci (combined in seven different multiplexes) and new multiplexing techniques for seven existing loci (Boudjemadi *et al.* 1999) described here were tested in vivi- and oviparous clades of the European Common Lizard, *Zootoca vivipara.* Thirty-three of the new and all seven old markers amplified in all samples. ZV14 only amplified in the oviparous individuals belonging to Clade B, but not in the viviparous Clade D. Because Clade A is strongly divergent (Cornetti *et al.* 2015a) in addition to the fact that we tested the amplification of the newly developed microsatellite markers in only one individual belonging to Clade A, it may be interesting to test the effectiveness of the new microsatellite markers in this clade further. Previous to this study, only seven microsatellite loci were available for this species (Boudjemadi *et al.* 1999, Remon *et al.* 2008, Molecular Ecology Resources Primer Deveolpment Consortium 2011) and their amplification protocol was not optimized. The loci and protocols we developed provide strong, economical tools for evolutionary and population genetic studies, including the identification of population structure and management/conservation units, the estimation of gene flow, effective population sizes, and for other applications including cost-efficient paternity analyses.

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