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TECHNICAL NOTE

New polymorphic microsatellite markers of the endangered meadow viper (*Vipera ursinii*) identified by 454 high-throughput sequencing: when innovation meets conservation

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Abstract The Next Generation Sequencing (pyrosequencing) technique allows rapid, low-cost development of microsatellite markers. We have used this technology to develop 14 polymorphic loci for the endangered meadow viper (*Vipera ursinii*). Based on 37,000 reads, we developed primers for 66 microsatellite loci and found that 14 were polymorphic. The number of alleles per locus varies from 1 to 12 (for 30 individuals tested). At a cost of about 1/3 that of a normal microsatellite development, we were able to define enough microsatellite markers to conduct population genetic studies on a non-model species.

Keywords Conservation genomics · Microsatellite · *Vipera ursinii* · 454 sequencing

Introduction

The recent advent of next-generation, high-throughput sequencing technology and the improvement of more traditional techniques has allowed biologists to venture into the study of the population genetic structure of non-model organisms, which was previously so time-consuming and expensive (Abdelkrim et al. 2009; Santana et al. 2009) that a population study of rare and/or cryptic species was rarely attempted. Paradoxically, the importance of studying habitat fragmentation, gene flow and hybridization in such species is higher due to their higher sensitivity to environmental perturbations (e.g. Spielman et al. 2004).

An array of molecular tools has been useful in conservation genetics and some of the most commonly used nowadays are microsatellite markers (Frankham et al. 2002; Goldstein and Schlötterer 1999). In the field of reptile conservation genetics, only a few microsatellite markers for a limited number of species (34; based on GenBank and Mullin and Seigel 2009) are available out of the 3149 snake species listed by the EMBL Reptile Database. Vipers are the most intensively studied group in Europe but specific primers have only been developed for one of them (*V. berus*: Carlsson et al. 2003; Ursenbacher et al. 2009).

The meadow viper (Bonaparte, 1835) is a widespread palaearctic Viperidae ranging from southeastern France to northwestern China. Nonetheless, this species is one of the most threatened snake species in Europe owing to its highly fragmented habitat (Ferchaud et al. 2011). This species is classified as endangered (EN) on the IUCN Red List of Threatened Species, strictly protected under CITES (Appendix I and II) and the Bern Convention. Scientific research has focused on different aspects, including population dynamics (Baron et al. 1996; Ferrière et al. 1996), cohabitation with other species (Agrimi and Luiselli 1992; Luiselli et al. 2007), genetic diversity reduction (Ujvari et al. 2002, 2005) and population fragmentation (Ferchaud et al. 2011). However, no specific microsatellite locus has yet been isolated for this snake.

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Here, we describe the characterization of 15 microsatellite loci for *V. ursinii* using next generation pyrosequencing methods and tested their polymorphism in a French population.

Materials and methods

We harvested DNA from a stillborn specimen sampled from the population 1 mentioned in Ferchaud et al. (2011). The extraction was done with a Qiagen DNeasy kit and DNA ($\approx$10–15 ng) was sent to Microsynth AG (Balgach, Switzerland) for random 454 pyrosequencing and genomic library building. The genomic data was screened for potential microsatellites using MSATCOMMANDER v0.8.2 (Faircloth 2008) and a final selection was based on the length and the homogeneity of their repetitions. Two primers were designed for each selected microsatellite and the results of the PCR were examined by electrophoresis in a 1.5% agarose gel. A first selection of potential polymorphic loci was done with a QIAxcel machine (Qiagen, Hombrechtikon, Switzerland), using a QIAxcel DNA high-resolution kit (Qiagen). Subsequently, polymorphism of the microsatellite loci was tested for 30 samples of the population 3 (Ferchaud et al. 2011) with an ABI Prism 3130XL sequencer. The null allele occurrence was tested for each locus using MICRO-CHECKER v2.2.3 (Van Oosterhout et al. 2004). Observed (H_o) and expected (H_e) heterozygosity, deviation from Hardy–Weinberg Equilibrium (HWE) and genetic disequilibrium were calculated for each genetic marker using FSTAT v2.9.3 (Goudet 1995).

Results and discussion

The 454 sequencing provided 37,227 reads and MSTATCOMMANDER selected 3228 microsatellite loci (Table 1). Similar numbers of di-, tri- and tetranucleotide microsatellites were detected with a higher number of repetitions for dinucleotides (mean, minimum–maximum repetitions 12.8, 6–193; 8.64, 4–27; and 8.10, 4–42, respectively). We designed primer pairs for 66 microsatellite loci, but PCR products were obtained from only 44 microsatellite loci. A first test of polymorphism with the QIAxcel system suggested that 15 markers could be polymorphic.

Finally, primer pairs were combined to allow the amplification of all loci in five multiplex PCR reactions in order to amplify up to 4 loci in the same 10 µl PCR reaction (PCR conditions are given in Table 2). The number of alleles per locus ranged from 1 (Vu11 and Vu36) to 12 (Vu58) with expected heterozygosity values in the range 0.000–0.859 (Table 1). Only 1 (Vu52) microsatellite loci tested showed evidence of null alleles and HWE deviation. No evidence of genetic disequilibrium was detected.

The microsatellite markers developed in this work will be useful for the study of gene flow and inbreeding in the meadow viper. Based on the high throughput available

Table 1 Summary of the methodological test used to develop microsatellite markers from random sequencing using a next generation sequencer (454 pyrosequencing) and the proportion of usable microsatellite loci at each step

Step	No. microsatellite loci	Remarks
Random sequencing on 1/16th plate of a 454 sequencer (Roche)	Reads: 37,227	Mean length: 316.2 bp
Selection of microsatellites using MSATCOMMANDER v0.8.2 (Faircloth 2008)	Microsatellite loci: 3228 (but only 2462 for which potential primers could be designed)	Dinucleotides: 762 Trinucleotides: 780 Tetranucleotides: 920
Secondary selection of microsatellite loci with a long and stable number of repeats	Microsatellite loci: 66	Dinucleotides: 25 Trinucleotides: 21 Tetranucleotides: 20 This selection was subjective and numerous additional microsatellite loci could have been tested
Amplification and control of PCR product on agarose gel	Microsatellite loci: 44	After a few PCR tests with different temperatures and $MgCl_2$ concentration, we were unable to amplify 22 microsatellite loci with a clear and repeatable signal
Polymorphism test with QIAxcel system (Qiagen)	Microsatellite loci polymorphic: 15 of the 44	
Polymorphism test with capillary sequencer	Microsatellites polymorphic in the population 3 (Ferchaud et al. 2011): 13 of the 15	One non-polymorphic microsatellite locus in population 3 is polymorphic in other populations (Ferchaud pers. comm.)

Table 2 Characteristics of 15 microsatellite loci in *Vipera ursinii* tested with 30 samples of one population (population 3; Ferchaud et al. 2011)

Locus	Primer pair sequence (5′–3′)	Repeated motif	Anealing temperature (°C)	$MgCl_2$ concentration (mM)	Multiplex reaction	Length (bp)	Number of alleles	H_E	H_O	Occurrence of null alleles
Vu39	GGCATGGTGGAAGGGACC CGTTGTATTTGCTTGTTCCTGC	$(AAT)_{14}$	60	3.5	Reaction 1	172	3	0.444	0.412	No
Vu38	GGAAATCTGGTTGGAGCCCCC GCACTGTCTGTCACACC	$(AC)_{21}$	60	3.5	Reaction 1	173	4	0.635	0.559	No
Vu36	TCTGTGGGCTAACACCTGC TCTCCAGTTCATGTACCAGC	$(GT)_{19}$	60	3.5	Reaction 1	174	1	0.000	0.000	No
Vu29	GTAGGAGTCACTTGCTC GTTCATGGTCCTGTCCCTTTGG	$(CTA)_{13}$	60	3.5	Reaction 1	189	5	0.795	0.824	No
Vu55	GGTTGTGGATCTCTGACG GAGAACTCCCAACTCCAGGC	$(CAT)_{10}$	60	3.5	Reaction 2	154	2	0.505	0.529	No
Vu58	GCTGTCTAGTTTAATGGACCTGTC GAAGTTGAGAGGCTAATAAATCCAG	$(TCTA)_{16}TCA (TCTA)_2$	60	3.5	Reaction 2	190	12	0.859	0.912	No
Vu18	TGTTGCTGCCCATCTTGTC TGTCAAATATCAAGTCAGTTGCC	$(CT)_4TT(CT)_{10}$	60	3.5	Reaction 2	274	2	0.210	0.235	No
Vu57	GGAGTTGAAACAGTGTATGTCCAG CCAGGGCGAATAGTCCTC	$(CA)_{18}$	60	3.5	Reaction 2	172	8	0.831	0.882	No
Vu26	TAGGGTTTCCTGCCTGAGC CCTGGGTTACAAAGTCTCC	$(CAT)_9$	60	3.5	Reaction 3	238	2	0.211	0.176	No
Vu42	GGTTAATCAATCACGATAGCACCTCCG CACAGAGCAACATCTGCCACAG	$(AAC)_7$	60	1.5	Reaction 3	178	2	0.349	0.324	No
Vu32	TCTTTGGCTGCTTCAAGATTAG GCAGTAAGATGGAATTGG	$(CA)_{18}$	50	1.5	Reaction 4	152	3	0.615	0.588	No
Vu16	TCATTCTGGCTCTAATACCACATC GGAGGAAATGAGAAGACTGGC	$(ATTT)_{13}$	55	1.5	Reaction 4	181	4	0.626	0.588	No
Vu11	GCTTCCAGGCAGAAGGCG TTCCCTTTGTCCACCCAGC	$(AGG)_{10}$	55	1.5	Reaction 4	169	1	0.000	0.000	No
Vu52	GTATAGGGTGTCCTCC CCAGAGTCTGTGAGGAAGCCC	$(ATG)_{16}$	60	3.5	Reaction 5	192	4	0.703	0.176	Yes
Vu4	CAACTCGCAGCCAAACC TCGCCATCACTGGTACAGG	$(GAT)_9$	60	3.5	Reaction 5	153	3	0.258	0.294	No

using next generation sequencing, the rapid development of numerous microsatellite loci can be achieved with limited cost (in this study, the cost was about 1/3 that of a normal order). In addition, this method can provide hundreds of polymorphic primers (in our case, 21% of the developed loci were polymorphic) for non-model organisms. Consequently, we recommend that scientists interested in population genetics use this methodology to reduce developmental costs of new microsatellite markers for non-model species.

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