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TECHNICAL NOTE

# Thirteen polymorphic microsatellite markers for the European green toad *Bufo viridis viridis*, a declining amphibian species

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**Abstract** We report 13 new polymorphic microsatellite markers for the European green toad *Bufo viridis viridis* (*B. viridis* subgroup), a declining amphibian from Central, Southeastern and Eastern Europe. Diversity at these loci estimated for 19 individuals ranged from two to ten alleles. Most of these primers also cross-amplify in related West-Mediterranean green toad species (*Bufo balearicus*, *B. siculus* and *B. boulengeri*). These microsatellites will be useful for conservation genetics of threatened *Bufo viridis viridis* populations and evolutionary studies of green toad taxa in secondary contact to examine hybridization.

**Keywords** *Bufo viridis viridis* · Polymorphic microsatellites · Population genetics · Conservation research

## Introduction

Green toads are widespread in the Western Palearctics (Stöck et al. 2006). They comprise a monophyletic radiation of at least twelve major haplotype groups, several of which occur in Europe (for overview: Stöck et al. 2008 incl. refs.). The mitochondrial haplotype group that occurs at the type locality (Vienna) of *Bufo viridis viridis* (Laurenti 1768), is distributed from Crete, across Western Greece, West of the Carpathians into Central Europe, where it reaches its most Western range border approximately at the Rhine River. Another range part lies in Eastern Russia and reaches further East into northern Kazakhstan. Several taxa of the *B. viridis* subgroup are under threat including the Central European taxon *B. viridis viridis* (Podloucky and Manzke 2003), and the lineage distributed around the Baltic Sea, *B. variabilis*. At their Western range part both are considered rare and "threatened by extinction" or "strongly threatened", and even "extinct" in Switzerland (Grossenbacher 2003). This threat resulted in European legislation, and today "green toads" are generally protected according to Appendix 2 of the Berne Convention (1979), so far ratified in 45 countries, and according to Appendix IV of the Habitats Directive (1992). Species protection plans have been or are going to be implemented (e.g. Plan National 2010; ref. in Stöck et al. 2008). Besides major threats from industrial agriculture, traffic and its infrastructure, general habitat destruction and the potential threat from chytridomycosis (e.g. Kilpatrick et al. 2010), habitat fragmentation plays a role. As anurans that react opportunistically to changing breeding conditions, their great mobility requires connected habitats for viable metapopulations. Protection should be accompanied by research designed to test efficiency of measurements (e.g. population genetics). Microsatellites have so far been published for Western Mediterranean and Central Asian green toads (Colliard et al. 2009; Stöck et al. 2010), but they have not been available for *Bufo viridis viridis*.

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**Table 1** Thirteen new microsatellites for *Bufo viridis viridis*

| Locus | GenBank | Used label | Primer sequences | Repeat motif | Na | Allele size range (bp) | Sampling locality | *Ho* | *He* |
|---|---|---|---|---|---|---|---|---|---|
| Bcal$\mu$10 | AF267239 | FAM | F: ATGCTTTCATTGCTGGAGAGG | $(AG)_n$ | 2 | 149–156 | *Santa Croce* | 0.14 | 0.14 |
| | | | R: GCCCCCTTCCCTGTACCAAG | | | | *Cusignana* | 0.00 | NC |
| BaturaC124 | FJ613130 | HEX | F: CCCCATAACAAACTGCTGATA | $(TACA)_n$ | 2 | 160–163 | *Santa Croce* | 0.29 | 0.44 |
| | | | R: CTTGCTACAGGGCACTACAAC | | | | *Cusignana* | 0.00 | NC |
| BaturaC107 | FJ613131 | FAM | F: ACGGTGTGGATTCCTTTAG | $(TACA)_n$ | 2 | 223–227 | *Santa Croce* | 0.00 | NC |
| | | | R: AGCAGCATACAACAGGTGA | | | | *Cusignana* | 0.42 | 0.52 |
| BaturaC218 | FJ613134 | ATTO | F: GCATACCTTGGTCAATGATG | $(TAGA)_n$ | 10 | 155–195 | *Santa Croce* | 1.00 | 0.91 |
| | | | R: CAGGGAGAGCAGGCTTTA | | | | *Cusignana* | 1.00 | 0.83* |
| BaturaC201 | HQ386137 | HEX | F: AGGACCCAGGATTTCCAT | $(TCTA)_n$ | 8 | 91–119 | *Santa Croce* | 0.71 | 0.86 |
| | | | R: GCTTCTACCAAAGACTGTTCC | | | | *Cusignana* | 0.75 | 0.77 |
| BaturaC223 | FJ613135 | ATTO | F: CAGAGGTCAAGAGGGAGAAG | $(TCTA)_n$ | 4 | 171–203 | *Santa Croce* | 0.57 | 0.58 |
| | | | R: GGCACCACATCCTGATTAG | | | | *Cusignana* | 0.42 | 0.42 |
| BaturaC203 | FJ613132 | HEX | F: TTGAGGTCCACTAACCACTGTA | $(TAGA)_n$ | 7 | 201–227 | *Santa Croce* | 0.86 | 0.87 |
| | | | R: AGGATTGCCTGAAAACATTTAC | | | | *Cusignana* | 1.00 | 0.82 |
| BaturaC224 | HQ386138 | ATTO | F: TTTGGAAGGAAAGCATCTG | $(TAGA)_n$ | 10 | 161–213 | *Santa Croce* | 0.86 | 0.80 |
| | | | R: TCATCAACCTGGGATAATACTG | $(TAATA)_n$ | | | *Cusignana* | 0.92 | 0.88 |
| BaturaD105 | FJ613136 | FAM | F: AATGCAGAGATGCCATGTG | $(TAGA)_n$ | 5 | 182–207 | *Santa Croce* | 0.29 | 0.47 |
| | | | R: TTGGATGATCTTCCTTTCAAAG | | | | *Cusignana* | 1.00 | 0.76 |
| BaturaD210 | HQ386139 | FAM | F: GGCCAATGAAAGAGGAAC | $(TACA)_n$ | 2 | 174–178 | *Santa Croce* | 0.14 | 0.14 |
| | | | R: GCTTCGCTTATCCCTAGTTAC | | | | *Cusignana* | 0.08 | 0.08 |
| BaturaD5 | FJ613138 | HEX | F: CCTCTTTTACCTCTGACAGTGC | $(TCTA)_n$ | 3 | 113–152 | *Santa Croce* | 0.50 | 0.62 |
| | | | R: ACAGGCATGAAAACCATTGA | | | | *Cusignana* | 0.36 | 0.31 |
| BaturaD106 | FJ613137 | FAM | F: CCATTCCGAAGATGATAACA | $(GATA)_n$ | 4 | 210–224 | *Santa Croce* | 0.43 | 0.36 |
| | | | R: CACGCAAAAAATACAGATGAC | | | | *Cusignana* | 0.58 | 0.63 |
| BaturaC205 | FJ613133 | HEX | F: TCATCATCATCCCTCTCTATG | $(TCTA)_n$ | 6 | 172–256 | *Santa Croce* | 0.86 | 0.79 |
| | | | R: CCAAAGCAAAGTCAGTAACAG | | | | *Cusignana* | 0.91 | 0.72 |

*GenBank* accession number, *Used label* ABI-compatible fluorescent dye (forward primer labelled), *Ho* observed heterozygosity, *He* expected heterozygosity, *NC* He not calculated when Ho = 0

* Locus significantly deviates from Hardy–Weinberg expectations after Bonferroni correction

## Materials and methods

As reported previously (Colliard et al. 2009), microsatellite markers were obtained from a genomic library produced by the Genetic Identification Services, (www.genetic-id-services.com, DNA from *Bufo baturae*, voucher: ZMB 58770, Pasu, Karakoram, 36°29′20.0″N, 74°52′47.6″E, a related species from Pakistan). We also tested one primer pair, developed originally for *Bufo calamita* (Rowe et al. 2000) that has successfully been used in other green toads.

Genomic DNA was extracted from ethanol preserved muscle, tail tips (tadpoles) and frozen buccal swabs (QIAGEN DNeasy kit). Primers were tested by performing polymerase chain reactions (PCR) in 10 µl reactions, containing 1 µl DNA (10 ng/µl), 1 µl 10× PCR buffer (15 mM $MgCl_2$), 0.2 µl dNTPs (25 mM), 0.2 µl each primer (10 µM); forward fluorescently labeled), 0.2 µl additional $MgCl_2$ (25 mM), and 0.1 µl QIAGEN *Taq* (5 U/µl). Amplifications were carried out on GeneAmp PCR System 9700 (ABI; 3 min at 94°C, 38× (1 min at 94°C, 1 min at 50°C, 1 min at 72°C), 4 min at 72°C). Fragment analyses were performed (ABI PRISM 3100 sequencer) and allele sizes scored using the size standard ROX-350 (GeneMapper 4.0, AppliedBiosystems, Inc.).

To assess variability and genetic diversity in natural populations, we genotyped 19 *Bufo v. viridis* from two neighboring localities in northern Italy (Santa Croce, 45°51′0.32″N, 12°9′4.02″E, n = 7; Cusignana, 45°47′0.35″N, 12°11′38.98″E, n = 12; no larger sample size was available). Sequencing of the mtDNA d-loop (data not shown) showed our test populations to exhibit no signs of introgression from *B. balearicus* whose range borders to the south of the Po valley (Colliard et al. 2010). All 13 loci were tested for polymorphisms and deviations from

Hardy–Weinberg equilibrium using Arlequin 3.11 (Excoffier et al. 2005). We also calculated differentiation (Fst) and inbreeding (Fis) coefficients (Arlequin), tested for linkage disequilibrium (Fstat; Goudet 1995) and checked for null alleles (Micro-Checker 2.2.3; van Oosterhout et al. 2004).

## Results

Thirteen microsatellites (Table 1) could be successfully amplified and turned out to be polymorphic in *Bufo v. viridis* from the two test populations. Ten of them have been shown to cross-amplify in the related species *Bufo balearicus*, *B. siculus*, and *B. boulengeri* (for details: Colliard et al. 2009).

Our two test populations were significantly differentiated (Fst: 0.104, $P < 0.01$). However, inbreeding coefficients (Fis) were not significant and all loci but one (C218 in Cusignana) met Hardy–Weinberg expectations in both sampling areas. Furthermore, we neither detected evidence for null alleles, large allelic dropout, or scoring errors due to stuttering. Allele numbers ranged from 2 to 10, with some loci being highly polymorphic (allelic diversity $Na > 6$) as in related species (C205, C218; Colliard et al. 2009). We did not detect significant linkage disequilibrium between our loci in any population after Bonferroni corrections.

The microsatellites primers presented here will be useful to assess regional genetic structures of *Bufo viridis viridis* populations for conservation and other purposes. In addition, these markers will also be applicable to conduct population genetic studies in contact zones involving *Bufo viridis* and related taxa (e.g. *B. variabilis*.)

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