What remains from a 454 run: estimation of success rates of microsatellite loci development in selected newt species (*Calotriton asper*, *Lissotriton helveticus*, and *Triturus cristatus*) and comparison with Illumina-based approaches

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Abstract
The development of microsatellite loci has become more efficient using next-generation sequencing (NGS) approaches, and many studies imply that the amount of applicable loci is large. However, few studies have sought to quantify the number of loci that are retained for use out of the thousands of sequence reads initially obtained. We analyzed the success rate of microsatellite loci development for three amphibian species using a 454 NGS approach on tetra-nucleotide motif-enriched species-specific libraries. The number of sequence reads obtained differed strongly between species and ranged from 19,562 for *Triturus cristatus* to 55,626 for *Lissotriton helveticus*, with 52,075 reads obtained for *Calotriton asper*. PHOBOS was used to identify sequences with tetra-nucleotide repeat motifs with a minimum repeat number of ten and high quality primer binding sites. Of 107 sequences for *T. cristatus*, 316 for *C. asper* and 319 for *L. helveticus*, we tested the amplification success, polymorphism, and degree of heterozygosity for 41 primer combinations each for *C. asper* and *T. cristatus*, and 22 for *L. helveticus*. We found 11 polymorphic loci for *T. cristatus*, 20 loci for *C. asper*, and 15 loci for *L. helveticus*. Extrapolated, the number of potentially amplifiable loci (PALS) resulted in estimated species-specific success rates of 0.15% (*T. cristatus*), 0.30% (*C. asper*), and 0.39% (*L. helveticus*). Compared with representative Illumina NGS approaches, our applied 454-sequencing approach on specifically enriched sublibraries proved to be quite competitive in terms of success rates and number of finally applicable loci.
Introduction

Microsatellite loci are still considered valuable tools for addressing basic questions in ecology, evolution, and behavior in nonmodel organisms, despite the fact that other molecular markers have become increasingly popular due to next-generation sequencing (NGS) approaches (e.g., genotyping or sequencing of single-nucleotide polymorphisms (SNPs)). Microsatellite loci are currently still the marker of choice for comprehensive analyses of population structure (e.g., Palo et al. 2004; Jehle et al. 2005, 2007; Dogaç et al. 2013), mating systems (e.g., Jones et al. 2002; Schmeller et al. 2005; Steinfartz et al. 2006; Jehle et al. 2007; Loyau and Schmeller 2012), landscape (Storfer et al. 2010) and conservation genetics (reviewed by Jehle et al. 2007; Loyau and Schmeller 2012). Moreover, the current impact of microsatellite loci as genetic tools is also illustrated by more than 4000 scientific studies that have been published in the past two years matching the query “microsatellite loci” in the Web of Science and by recent publications reporting the development of new loci (i.e., Prunier et al. 2012; Castoe et al. 2012a,b; Dobes and Scheffknecht 2012). Before the application of NGS in the process of developing microsatellite loci, the isolation and characterization of new loci was a costly and sometimes elaborate endeavor (e.g., Zane et al. 2002). However, by using NGS approaches on genomic libraries enriched for microsatellite motifs, the isolation process has become much simpler and more cost-effective (e.g., Abdelkrim et al. 2009). Normally, these approaches result in tens of thousands of sequence reads, which are expected to lead to a large amount of suitable microsatellite loci (e.g., Yang et al. 2012). However, the correlation between the initial number of sequence reads obtained and the number of usable polymorphic microsatellite loci may be low as the number of potentially amplifiable loci (PALs) is negatively influenced by many factors. These factors include sequence read quality (cut-off score values), motif length (type and number of repeat), and the presence, quality, and necessary length of the primer region, in addition to the amplification success and confirmed polymorphism of loci across the studied populations. Accordingly, systematic approaches to estimate success rates of microsatellite loci development for quite distinct taxa are important to finally obtain a sufficient number of applicable loci (e.g., Castoe et al. 2010, 2012a,b; Prunier et al. 2012). For example, in the copperhead snake (Agkistrodon contortrix), Castoe et al. (2010), isolated 4,564 PALs from 128,773 reads, but only found 80 tetra-nucleotide PALs (i.e., 0.062% of all reads) with more than 10 repeat units. In the alpine newt (Ichthyosaura alpestris), Prunier et al. (2012) obtained 1015 microsatellite motif-bearing sequence reads, with a final yield of 14 microsatellite loci from 61 tested primer pair combinations. Microsatellite development might be especially tedious in amphibians due to their large genome sizes and comparably low numbers of PALs, which have made development and isolation approaches in the past both cost- and time-intensive (e.g., Hendrix et al. 2010; Hauswaldt et al. 2012). Accordingly, NGS-based microsatellite loci development approaches should be efficient in obtaining a sufficient number of loci in such species (e.g., amphibians).

In this study, we used a 454-sequencing approach with enriched libraries to develop highly polymorphic tetra-nucleotide microsatellite loci for three distinct newt species within the family of Salamandridae (Calotriton asper, Lissotriton helveticus, and Triturus cristatus). We determined the success rate of our approach by estimating the number of PALs based on the number of usable polymorphic loci tested across several populations of each species and compared it with Illumina-based sequencing approaches of recently published studies. Furthermore, we tested the cross-amplification success rate of the developed loci for C. asper in the highly endemic and threatened species C. arnoldi, the Montseny brook newt (see Carranza and Amat 2005).

Materials and Methods

Study species

Triturus cristatus, the great crested newt (Fig. 1), is widely distributed from the United Kingdom to northern France, through southern Scandinavia to central Europe, and into a small part of the Balkans. The species is listed on the Habitats Directive of the European Union (92/43/EEC) and is threatened by exposure to fish, habitat loss, and habitat fragmentation (see Jehle et al. 2011; Denoël 2012; Denoël et al. 2013). Thus far, only eight applicable microsatellite loci for T. cristatus have been published (Krupa et al. 2002), which might be an insufficient number to reveal consistent results in population genetic analyses (e.g., SPOTG software; Hoban et al. 2013). For the two endemic mountain brook newt species of the genus Calotriton (C. asper, the Pyrenean brook newt and C. arnoldi, the Montseny brook newt, found in the northeastern Iberian Peninsula), no microsatellite loci have been reported thus far. Both species are endemic to comparable small ranges (especially C. arnoldi) and are habitat specialists that are adapted to high mountain brooks and have a cryptic life history. C. asper is listed as near threatened (NT), and C. arnoldi is listed as critically endangered (CR) according to the IUCN Red List v3.1 (http://www.iucnredlist.org/static/categories_criteria_3_1). Therefore, the development of microsatellite loci for these spe-
cies is an important contribution to efforts to better understand their ecology and evolution and will consequently assist in their conservation. *L. helveticus*, the palmate newt, is distributed throughout Western Europe and is in decline in some parts of Europe due to habitat loss and fragmentation (Denoël and Ficetola 2007). Our study adds additional microsatellite loci to the already existing set of eight loci for this species (Johanet et al. 2009).

### Sampling and DNA extraction

Tissue samples of *T. cristatus* were collected from two different populations in the Kottenforst near Bonn and in the Latumer Bruch in Krefeld (North Rhine-Westphalia, Germany) (Table 1). Tissue samples of *C. asper* were collected from four different sampling sites from the Spanish and French sides of the Pyrenees (Table 1). Samples of *C. arnoldi* were taken by clipping single toes or tail tips, with permission of the local authorities, and stored in 80% ethanol.

Total genomic DNA was extracted using the sodium-dodecyl-sulfate (SDS)-proteinase K/Phenol–Chloroform extraction method, after which it was stored in Tris-EDTA buffer (10 mmol/L Tris-HCl, 0.1 mmol/L EDTA, pH 8.0) and then used for all subsequent reactions. To start the enrichment procedure for the three distinct target species (i.e., *T. cristatus*, *L. helveticus*, and *C. asper*) with more or less similar amounts of genomic DNA, we estimated DNA concentrations of different individuals on a 1% agarose gel and selected those with a concentration of 100–200 ng/µL, as suggested by Glenn and Schable (2005).

### Enrichment of microsatellite loci and 454-sequencing

The enrichment protocol followed the selective hybridization method with minor modifications (Zane et al. 2002; Glenn and Schable 2005); and the enrichment procedure was performed separately for each target species. Genomic DNA was digested into approximately 500 bp fragments using *Rsa I* enzyme and *Xmn I* to avoid linker dimerization. Double-stranded linkers were annealed to both ends of the fragments to obtain a primer-binding site for subsequent PCR. Linker sequences were as follows: SimpleXL03_U: 5′-AAAACGTGCTGCGGAAGTACT-3′ and SimpleXL03_Lp 5′-pAGTCCGACGAC-3′. PCR was performed in 25 µL reactions to test whether annealing was successful; this PCR product was then used for the next step to increase the concentration of linker-ligated DNA. To capture DNA fragments containing microsatellite loci sequences out of all linker-ligated fragments, 50 µL of Streptavidin M-280 Dynabeads (Invitrogen, Carlsbad, CA, USA) was used. To enrich for tetra-nucleotide motif-bearing DNA fragments, biotinylated oligo probes and linker-ligated DNA fragments were mixed as described by Glenn and Schable (2005). For this step, the following oligo probes were used: (AAAGT)$_8$, (AGAT)$_8$, (ACAT)$_8$, (AAAT)$_8$, (AATC)$_6$, (ACAG)$_6$, (AAAC)$_6$, (AATC)$_6$, (ACAG)$_6$, (ACTC)$_6$, (ACTG)$_6$, (AATT)$_6$, and (ACCT)$_6$. PCR was performed to recover the microsatellite-enriched DNA fragments (Glenn and Schable 2005). After amplification, all samples were quantified using a Nanodrop spectrophotometer. Afterward, the samples were processed according to the cDNA Rapid Library Preparation Method Manual (Roche, Mannheim, Germany) beginning with step 3.3 and omitting step 3.4. Multiplex Identifier (MID) Adaptors for Rapid Libraries (Roche, Branford, CT) were ligated to the DNA fragments of each sample (*T. cristatus*: MID ACA-CTACTCGT, MID ACAGACACGT; *C. asper*: MID ACAGTAGACT, MID ACGCCCTCTAG; *L. helveticus*: MID ACCTACACT, MID ACCTACTGCT). The DNA fragments were cleaned and subsequently quantified using an Agilent 2100 Bioanalyzer. As a final step, the individual samples were combined into a DNA library pool, which was run on an Agilent 2100 Bioanalyzer prior to emulsion PCR and sequencing, as recommended by Roche. The library was not denatured prior to pipetting onto the washed capture beads (step 3.2.8, emPCR Method Manual – Lib-L SV, Roche, Branford, CT, USA). The library was subsequently sequenced on a 454 GS-FLX using Titanium sequencing chemistry.

| Species          | Population     | Geographic coordinates |
|------------------|----------------|------------------------|
| *Triturus cristatus* | Krefeld          | 6°39′17″E, 51°19′05″N  |
|                  | Kottenforst     | 7°31′13″E, 50°40′24″N  |
| *Calotriton asper* | Ibión Perraco  | 0°29′59″E, 42°38′20″N  |
|                  | Barranco Valdaga | 0°47′38″E, 42°51′29″N  |
|                  | Ibión d’Acherito | 0°42′25″E, 42°52′46″N  |
| *Lissotriton helveticus* | Basses            | 1°24′58″E, 42°46′04″N  |
|                  | Mas d’Ausse    | 3°19′34″E, 43°58′30″N  |
|                  | Campells North  | 3°34′15″E, 43°57′39″N  |
|                  | Bagnelade      | 3°21′42″E, 43°51′20″N  |
|                  | Coulet Northeast| 3°32′23″E, 43°49′15″N  |
|                  | Le Cros Ferme  | 3°22′10″E, 43°52′11″N  |
Estimation of microsatellite loci success rates for the different species

To estimate the number of PALs based on the numbers of polymorphic loci initially identified, we used steps A through I described below. (A) The PHOBOS software version 3.3.11 (Mayer 2007) was used to assign obtained sequence reads to a target species on the basis of the species-specific MID tags. PHOBOS was also used to identify sequence reads containing noninterrupted stretches of at least ten tetra-nucleotide repeat motifs. (B) Selected sequences were retained by PHOBOS only when the flanking region on each side of the repeat motif was at least 25 bp long. These sequences were then assessed by eye for their general suitability for primer design, that is, sequences with more than five repetitive nucleotides at a stretch were removed. (C) Score values, indicating the quality of each retrieved sequence, were assessed, and sequences with values below 20 (of a maximum score of 40) were discarded from further analysis. (D) We designed primers using the software Primer 3 (version 0.4.0, Rozen and Skaletsky 2000) with default settings (i.e., an optimum primer temperature of 60°C). (E) We tested all primer pairs for amplification success and subsequent degree of polymorphism and heterozygosity in at least 21 individuals. In a first step, a universal M13-tail was attached to the forward primer as a cost-reducing method (Schuelke 2000). (F) Only for the polymorphic microsatellite loci in *C. asper* and *T. cristatus*, we designed primers without the M13-tail but with fluorescence labeling. These primers were tested in a 10 µL of Type-it multiplex PCR (Qiagen) containing 1 µL of DNA for up to 902 individuals per microsatellite locus. Primers were combined in either three (*C. asper*) or two (*T. cristatus*) multiplex mixes, supplemented by one mix of previously published loci for *T. cristatus* (see Krupa et al. 2002). Applied PCR parameters were as follows: (1) an initial Taq polymerase activation step of 5 min at 95°C, (2) 30 s at 94°C, (3) 90 s at an annealing temperature of 60°C, (4) 60 s extension at 72°C, (5) steps 2–4 repeated for 30 cycles, and (6) a final extension phase of 30 min at 60°C. PCR products were diluted in 200 µL of water, and 1 µL of diluted product was added to 19 µL of Genescan 500-LIZ size standard (Applied Biosystems) prior to analysis on an ABI 3730 96-capillary automated DNA sequencer. (G) Analysis of the microsatellites was performed using GENEMARKER software (version 1.95, SoftGenetics, State College). Tests for null alleles, deviations from Hardy–Weinberg equilibrium, and linkage disequilibrium were performed using CERVUS (version 3.0.3, Field Genetics Ltd., Marshall et al. 1998). (H) Primer pairs for *C. asper* were also tested for cross-amplification success in *C. arnoldi* using the *C. asper* multiplex mixes with 41 *C. arnoldi* samples originating from two different sectors. Six individuals were tested from sector 1, and 36 individuals were tested from sector 2; PCR conditions were as described for *C. asper*. (I) The estimation of the number of PALs for each species was performed by extrapolating the number of suc-

| 454-approach (this study) | Number of sequence reads | Tetra-nucleotides >10 repeats | Tetra-nucleotides >10 repeats + priming sites (>25bp) | NTPP | SIPL | Estimated PALs – success rate (%) |
|--------------------------|--------------------------|-------------------------------|-------------------------------------------------|------|------|----------------------------------|
| *T. cristatus*            | 19,562                   | 936                           | 107                                             | 41   | 11   | 29 (0.15)                        |
| *C. asper*                | 52,075                   | 1083                          | 316                                             | 41   | 20   | 154 (0.30)                       |
| *L. helveticus*           | 55,626                   | 1434                          | 319                                             | 22   | 15   | 217 (0.39)                       |

| Illumina approach         | Number of sequence reads | PAL_FINDER Di, tri, tetra, penta, hexa² | Number of tested loci | Number of polymorphic loci | Estimated PALs – success rate (%) |
|--------------------------|--------------------------|----------------------------------------|-----------------------|---------------------------|----------------------------------|
| *Cyprinodon julimes*/C. pachycephalus¹  | 5 million                | 1590 loci                              | 48                    | 25 (C. julimes)/17 (C. pachycephalus) | 828 (0.016)¹                    |
| *Leptodea leptodon*³      | 5 million                | 3905 loci                              | 48                    | 16                        | 1301 (0.026)                     |
| *Ambystoma annulatum*⁴    | 5 million                | 749 loci²                              | 150                   | 22                        | 109 (0.00219)                    |

¹Carson et al. (2013).  
²Castoe et al. (2012a,b).  
³O’Bryhim et al. (2012).  
⁴Peterman et al. (2013).  
⁵Note that the study of Peterman et al. (2013) only analyzed tetra- and penta nucleotide motifs.
cessfully isolated polymorphic loci (SIPL) in relation to the number of tested primer pairs (NTPP). That rate was then used to calculate the number of PALs for sequence reads that passed the criteria described in (B) and (C). Here, an example calculation is provided. We isolated 11 SIPL of 41 NTPP for T. cristatus, resulting in a SIPL/NTPP ratio of 26.83%. Extrapolating this ratio to the 107 sequences that passed criteria (B) and (C) resulted in 29 PALs. Thus, the overall success rate (PALs/number of sequences) for T. cristatus was 0.15% (see Table 2).

Results

The 454-sequencing resulted in a total of 127,263 sequence reads from one quarter run for the enriched libraries of the three species. Sequencing results and success rates of microsatellite loci development are summarized in Table 2. The species classification by MID tag identification of all sequences performed by PHOBOS (Methods, step A) led to 19,562 sequences for T. cristatus (15.37% of all sequences), 52,075 sequences for C. asper (40.92%), and 55,626 sequences for L. helveticus (43.71%). PHOBOS identified 936 sequences (4.78% of all T. cristatus reads) containing a noninterrupted stretch of ten or more tetra-nucleotide repeat motifs in T. cristatus, 1083 (2.08% of all C. asper reads) sequences in C. asper, and 1434 (2.58% of all L. helveticus reads) sequences in L. helveticus. The scan for suitable PCR priming sites (i.e., 25 bp in minimum in each direction) performed in PHOBOS (Methods, step B) resulted in 107 sequences for T. cristatus, 316 sequences for C. asper, and 319 sequences for L. helveticus (Table 2). The assessment for nonrepetitive flanking regions and the exclusion of sequences with score values of poor quality (Methods, steps C and D) led to 41 ordered primer pairs for T. cristatus, 41 for C. asper, and 22 for L. helveticus. In T. cristatus, 14 of 41 tested loci were polymorphic (Methods, step E) and were sequentially labeled with fluorescence dyes. In C. asper, 21 of the 41 tested loci were polymorphic, while 15 of the 22 tested loci were polymorphic in L. helveticus. While the 15 L. helveticus loci were analyzed without any additional labeling (Methods, step G), the Type-it multiplex PCR of the T. cristatus and C. asper loci resulted in the detection of 13 (T. cristatus) and 21 (C. asper) polymorphic loci (Methods, step F). Validation of these candidate loci (Methods, step G) yielded 11 loci for T. cristatus, 20 loci for C. asper, and 15 loci for L. helveticus (summarized in Tables 3–5). Detailed information on the number of tested individuals per population, the expected and observed heterozygosity, tests for deviations from Hardy–Weinberg equilibrium with a Bonferroni correction, linkage disequilibrium, and report of null alleles is provided for each species in supplementary Tables S1–S3. The test for cross-amplification success of C. asper primer pairs in C. arnoldi (Methods, step H) resulted in 10 polymorphic loci (Table 4). The SIPL/NTPP ratios (Methods, step I) for T. cristatus, C. asper, and L. helveticus were 26.83%, 48.78%, and 68.18%, respectively. The adoption of this ratio to calculate the number of PALs resulted in 29 tetranucleotide PALs for T. cristatus, 154 for C. asper, and 217 for L. helveticus. The PALs/number of sequences ratio was 0.15% for T. cristatus, 0.30% for C. asper, and 0.39% for L. helveticus (Table 2).

Discussion

Comparison of success rates of NGS-based microsatellite loci development

For many nonmodel organisms, the de novo development of microsatellite loci has been enormously improved by the implementation of NGS approaches. In the past, the development of microsatellite loci for amphibian species using classic cloning approaches was rather time-consuming and costly, possibly due to their large genomes, which are comparably rich for long repetitive DNA stretches resembling in part microsatellite motifs (e.g., in salamanders; see fig. 1.1 in Steinfartz 2003). Based on our experiences, only the use of enrichment procedures (see Zane et al. 2002) enabled the development of a sufficient number of polymorphic microsatellite loci applicable for genetic studies in various amphibian target species (e.g., Steinfartz et al. 2004; Hauswaldt et al. 2008, 2012; Hendrix et al. 2010). Here, we employed an NGS approach to sequence genomic sublibraries enriched for tetra-nucleotide motifs of three newt species, for which only a limited number (in the case of T. cristatus and L. helveticus) or no (C. asper) loci had previously been available. Although NGS approaches have certainly improved the development of new microsatellite loci, many studies using this approach do not report actual success rates of applicable loci compared with the large number of sequencing reads initially obtained (e.g., Gardner et al. 2011). This might lead to the impression that, by using NGS approaches, very high numbers of new loci can easily be developed. However, the pure occurrence of a microsatellite locus motif in a sequencing read does not guarantee that this locus can be developed into an applicable polymorphic locus for subsequent genetic analyses. Low sequence read quality, motif length (type and number of repeat), and the presence and appropriate length of the surrounding primer region are major factors that can dilute the fraction of potentially amplifiable loci (PALs) enormously. Our aim was therefore to develop high-quality tetra-nucleotide motif-bearing microsatellite loci with a demonstrated utility for subsequent genetic analyses of respective target
species. Based on the number of obtained polymorphic loci, we extrapolated species-specific success rates, which were found to be quite low, that is, below one percent (see Table 2).

Although our success rates seem to be unexpectedly low, they are in line with numerous other studies, from which success rates are reported or can be calculated. Using 454-sequencing technology but no specific enrichment protocol, Castoe et al. (2010) identified 80 tetra-nucleotide PALs (0.06% of total sequencing reads) with more than 10 motif repeats for the copperhead snake (*Agkistrodon contortrix*). In a parallel study in the coral snake (*Micrurus fulvius*), they were able to identify 54 tetra-nucleotide PALs (0.20% of total sequencing reads) with more than 10 motif repeats (Castoe et al. 2012a). Our success rate of 0.15% for *T. cristatus* (29 PALs) was similarly low. In contrast, the estimated 154 PALs for *C. asper* with a success rate of 0.30% and 217 PALs for *L. helveticus* with a success rate of 0.39% were considerably higher. When applying more relaxed comparison criteria between studies, 454-application of the full set of 17 applied microsatellite loci for *Triturus cristatus*, including the 11 newly developed primer pairs from this study (highlighted in bold) along with six previously published loci (Krupa et al. 2002). Loci are grouped by the multiplex combinations used for amplification. Information on the locus name, primer sequence, direction (F is forward, R is reverse), annealing temperature of the primer for PCRs, microsatellite motif, amplified fragment size range, number of alleles, and labeling dye are provided together with the accession number of the associated GenBank sequence.

**Table 3.** Characterization of the full set of 17 applied microsatellite loci for *Triturus cristatus*, including the 11 newly developed primer pairs from this study (highlighted in bold) along with six previously published loci (Krupa et al. 2002). Loci are grouped by the multiplex combinations used for amplification. Information on the locus name, primer sequence, direction (F is forward, R is reverse), annealing temperature of the primer for PCRs, microsatellite motif, amplified fragment size range, number of alleles, and labeling dye are provided together with the accession number of the associated GenBank sequence.

| Locus name | Primer sequence (5′-3′) | Annealing temp. (°C) | Repeat motif of cloned alleles | Size range of amplification product | Number of alleles | Fluorescence labeling | GenBank accession |
|------------|-------------------------|----------------------|-------------------------------|------------------------------------|------------------|----------------------|-------------------|
| Multiplex 1 |                         |                      |                               |                                    |                  |                      |                   |
| Tcri13     | F: GTATGGTTGCCAACAG     | 60°C                 | (GT)36 interrupted            | 93-131                             | 10               | FAM                  | AJ292500          |
|            | R: GATCCACACACACATATTAG |                      |                               |                                    |                  |                      |                   |
| Tcri27     | F: GATCCATATGAAATATAATAAAG | 60°C               | (GAAA)27 interrupted          | 241-288                            | 18               | FAM                  | AJ292517          |
|            | R: CAAGTTATATGATGCTTGG  |                      |                               |                                    |                  |                      |                   |
| Tcri29     | F: CGAGTGCCCAAGAACAG    | 60°C                 | (TTCA)22(CA)11 interrupted    | 289-340                            | 11               | NED                  | AJ292505          |
|            | R: GATCACATGCCCATATGG   |                      |                               |                                    |                  |                      |                   |
| Tcri35     | F: CCAACCTGTATGGCATTG   | 60°C                 | (GAAA)32 interrupted          | 200-234                            | 10               | NED                  | AJ292490          |
|            | R: GATCCACACACATATAAACAG|                      |                               |                                    |                  |                      |                   |
| Tcri36     | F: GATCATCTGAACTCTGCTG  | 60°C                 | (GAAA)36 interrupted          | 214-320                            | 24               | VIC                  | AJ292491          |
|            | R: ATACATCTATGGCTTGG    |                      |                               |                                    |                  |                      |                   |
| Tcri46     | F: CAAGTTCTCCTGAAAGCCAG | 60°C                 | (TTCT)33 interrupted          | 253-311                            | 15               | PET                  | AJ292494          |
|            | R: GTTCCTGCGACAAATAGGCTTC |                   |                               |                                    |                  |                      |                   |
| Multiplex 2 |                         |                      |                               |                                    |                  |                      |                   |
| Tc50       | F: CCGGATACATGCTTCTCGTT | 60°C                 | (ACTC)18                       | 177-268                            | 26               | PET                  | KF442195          |
|            | R: TCCAGTAAAATGCTGCTGCTG |                      |                               |                                    |                  |                      |                   |
| Tc52       | F: GGCTCTCGGAGAATGAGGAG | 60°C                 | (ATTG)17                       | 190-206                            | 6                | VIC                  | KF442196          |
|            | R: CCGTCATGTGTGCTGCTG   |                      |                               |                                    |                  |                      |                   |
| Tc66       | F: CTTGTGACACCCTGCAAA   | 60°C                 | (ATCC)18                       | 218-250                            | 8                | FAM                  | KF442197          |
|            | R: TGGTCTCATGAAAGGTCTGTCG |                     |                               |                                    |                  |                      |                   |
| Tc68b      | F: AAATGTGGCTCTCTGTGAGG | 60°C                 | (ATCC)24                       | 130-196                            | 13               | FAM                  | KF442198          |
|            | R: TCGAAGTGCATGTGCTGACT |                      |                               |                                    |                  |                      |                   |
| Tc70       | F: GGTTGCAAGGCACTTTATAT | 60°C                 | (ACAT)14                       | 216-232                            | 6                | VIC                  | KF442199          |
|            | R: TACTGGCTCTCTCCTCAAG  |                      |                               |                                    |                  |                      |                   |
| Tc81       | F: TTTAGTCCTCGCCGCTTCGAA | 60°C                | (AATC)13                       | 124-160                            | 10               | VIC                  | KF442200          |
|            | R: AGCGGAACTCTCCTCTATTG |                      |                               |                                    |                  |                      |                   |
| Multiplex 3 |                         |                      |                               |                                    |                  |                      |                   |
| Tc58       | F: ACAGGCCAGTGGCAAGAGAAG | 60°C                 | (AATC)7                        | 200-204                            | 2                | NED                  | KF442201          |
|            | R: CCAACCAGACACCCTTCTTC |                      |                               |                                    |                  |                      |                   |
| Tc69       | F: AGGTAGCCTTCGGCCACTAT | 60°C                 | (AGAT)13                       | 168-188                            | 6                | NED                  | KF442202          |
|            | R: GCTGATGCTCTGGGCAATTTA |                     |                               |                                    |                  |                      |                   |
| Tc71       | F: CCGCCAAATCAGCAATAATTA | 60°C                | (ACAT)11                       | 179-199                            | 5                | PET                  | KF442203          |
|            | R: AGTTGGGAAACACTGCTGGAAG |                      |                               |                                    |                  |                      |                   |
| Tc74       | F: TCTGTGACATGCTCTGATAGTGAAG |                    |                               |                                    |                  |                      |                   |
|            | R: TAGACACATTGAGACCTCTCAC |                       |                               |                                    |                  |                      |                   |
| Tc85       | F: GTTACGCTCAGCTGTTGGG  | 60°C                 | (AATC)11                       | 161-169                            | 3                | VIC                  | KF442205          |
|            | R: CCTCAAGACCTTGCTCTACG |                      |                               |                                    |                  |                      |                   |
based microsatellite loci development in the meadow viper resulted in only 14 applicable loci out of 37,000 sequence reads (0.037% success rate) and in a success rate of only 0.007% in the Asp viper (Geser et al. 2013) – both studies were performed without prior enrichment of genomic sublibraries. Accordingly, our obtained success rates are comparable quite high and seem to justify the applied enrichment procedure.

There is no doubt that Illumina sequencing is by far more cost-effective than 454-sequencing. Castoe et al. (2012b) suggest that Illumina-based microsatellite loci development is by far more effective than 454-based

| Multiplex 1 | Locus name | Primer sequence (5′–3′) | Annealing temp. (°C) | Repeat motif of cloned allele | Size range of amplification product | Number of alleles | Number of alleles in C. arnoldi | Fluorescence labeling | GenBank accession numbers |
|-------------|------------|--------------------------|----------------------|-------------------------------|-----------------------------------|-----------------|------------------------------|----------------------|------------------------|
| Ca1         | F: TGGAAACAGATGGCGTTGAA | (AGAT)_{16} | 60°C | 158–170 | 4 | 3 | FAM | KF442206 |
|             | R: TTCTGCAACCTCTCTGCTTCT | | | | | | | |
| Ca3         | F: CCATGCAATCTTTGAGGTTT | (AGAT)_{15} | 60°C | 246–288 | 5 | 6 | FAM | KF442207 |
|             | R: TTCAAGAGCAGTTGTCCAGGG | | | | | | | |
| Ca7         | F: ACCCTTACACACCCAAACC | (AGAT)_{16} | 60°C | 232–264 | 6 | 5 | NED | KF442208 |
|             | R: GTCTCCTGCTGCTCTTAAA | | | | | | | |
| Ca21        | F: AGGCTGTGACGAGCATGTTCC | (AGAT)_{15} | 60°C | 234–266 | 7 | 5 | VIC | KF442209 |
|             | R: GCAATGCTGACGCTTACACC | | | | | | | |

| Multiplex 2 | Locus name | Primer sequence (5′–3′) | Annealing temp. (°C) | Repeat motif of cloned allele | Size range of amplification product | Number of alleles | Number of alleles in C. arnoldi | Fluorescence labeling | GenBank accession numbers |
|-------------|------------|--------------------------|----------------------|-------------------------------|-----------------------------------|-----------------|------------------------------|----------------------|------------------------|
| Ca8         | F: AGAAGGAGGATCGACGCAGACA | (AGAT)_{13} | 60°C | 174–182 | 3 | 2 | FAM | KF442213 |
|             | R: GGAGGATCAATGTTGTTGGA | | | | | | | |
| Ca16        | F: GCACAAACATGATGGGTATGC | (AGAT)_{12} | 60°C | 119–131 | 4 | – | FAM | KF442214 |
|             | R: ACCGCATGCATGATAGTGCT | | | | | | | |
| Ca23        | F: CTTGCTGAAACCTCTATGG | (AGAT)_{14} | 60°C | 226–266 | 9 | – | PET | KF442215 |
|             | R: TTTCCTACACCTCTATCCTG | | | | | | | |
| Ca38        | F: CCTGTTAGGGAAGTAGTGGCA | (AATG)_{12} | 60°C | 166–178 | 3 | – | VIC | KF442216 |
|             | R: CTGGTGAGCATGCTTAT | | | | | | | |
| Us2         | F: TGGGCTGAAGGATTGAAAAA | (AGAT)_{17} | 60°C | 242–250 | 6 | – | VIC | KF442217 |
|             | R: CTCAAGCTGAGTGGTAGGTT | | | | | | | |
| Us3         | F: AAGTTTGTAGGTATGCATAATAGCC | (AGAT)_{16} | 60°C | 184–192 | 3 | – | NED | KF442218 |
|             | R: GGAAGCTGACGGTCCTGAGAGCA | | | | | | | |

| Multiplex 3 | Locus name | Primer sequence (5′–3′) | Annealing temp. (°C) | Repeat motif of cloned allele | Size range of amplification product | Number of alleles | Number of alleles in C. arnoldi | Fluorescence labeling | GenBank accession numbers |
|-------------|------------|--------------------------|----------------------|-------------------------------|-----------------------------------|-----------------|------------------------------|----------------------|------------------------|
| Ca5         | F: CGGTATTTGCTGTTGGATGG | (ACAT)_{10} | 60°C | 218–222 | 2 | – | VIC | KF442219 |
|             | R: TGCTAGTGGATACCTCTATCG | | | | | | | |
| Ca20        | F: CAGCGGTTAATACCATACAGGA | (AGAT)_{15} | 60°C | 200–232 | 10 | – | FAM | KF442220 |
|             | R: CCAACAGATCCCTCTCTCAACA | | | | | | | |
| Ca25        | F: CTTTGTGCTCTGTCAGTCC | (ACAT)_{14} | 60°C | 168–172 | 2 | – | PET | KF442221 |
|             | R: TTGCAGTGGATCTGTTGGA | | | | | | | |
| Ca29        | F: TCCATAAGCCTATTGTTGCG | (AATC)_{10} | 60°C | 246–258 | 4 | – | VIC | KF442222 |
|             | R: AGTGCACCTGCTCACTAGTG | | | | | | | |
| Ca30        | F: TCCATAAGCCTATTGTTGCG | (AATC)_{10} | 60°C | 108–120 | 3 | – | NED | KF442223 |
|             | R: GACCCCTATGGTTGTTGACG | | | | | | | |
| Ca32        | F: ACGAGCGAAGAGGATCACAGG | (ACAG)_{10} | 60°C | 148–200 | 4 | – | NED | KF442224 |
|             | R: CAGCCTATTGCTCTGTCAGC | | | | | | | |
| Ca35        | F: GGCGGCTTTACAAAGTGGTTACC | (AATC)_{10} | 60°C | 126–166 | 8 | – | FAM | KF442225 |
|             | R: CTGTCGCAAGGTAGAGGTTCA | | | | | | | |

Table 4. Characterization of the full set of 20 applied microsatellite loci for Calotriton asper. Loci are grouped by multiplex combinations used for amplification. Locus name, primer sequence, direction (F is forward, R is reverse), annealing temperature of the primer for PCRs, microsatellite motif, amplified fragment size range, number of alleles, labeling dye, and GenBank accession number are provided. The number of alleles of C. asper microsatellite loci detected in C. arnoldi cross-amplification is also provided; polymorphic loci are highlighted in bold.
In their comparative analysis, they obtain quite high success rates (called discrete PAL rate) ranging from 37–50% for both Illumina- and 454-based approaches, respectively. However, one important drawback of this study was that loci were not specifically tested for final performance and success rates might be therefore strongly overrated. As Illumina sequencing is now commonly applied for microsatellite loci development, we tried to estimate obtained success rates of three studies representative for quite diverse organisms such as fish (Carson et al. 2013), Bivalva (O’Bryhim et al. 2012) and salamanders (Peterman et al. 2013). Although Illumina sequencing resulted in higher number of suitable loci, final success rates were one order smaller than obtained success rates of the combined enrichment-454-sequencing approach (see Table 2). Also here, the enrichment for certain microsatellite loci motifs (e.g., tetra-nucleotides as in our approach)

| Locus name | Primer sequence (5’–3’) | Annealing temp. (°C) | Repeat motif of cloned allele | Size range of amplification product | Number of alleles | GenBank accession |
|------------|--------------------------|----------------------|-------------------------------|------------------------------------|------------------|-------------------|
| Lh1        | F: CAGCTGCAAGCGCAAGGAAG R: GTTCACACGGATTTGGTGG | 60°C | (AGTG)_{20} | 156–228 | 10 | KF442226 |
| Lh2        | F: TGCCAGGAGAGAGTTTCAT R: TGGGACCGAGGATGTAAGT | 60°C | (ATGT)_{12} | 154–226 | 6 | KF442227 |
| Lh6        | F: CTTGATATGCTCAGGAGGA R: GAAACTGCTCAGACCTCCT | 60°C | (ATAG)_{12} | 156–228 | 9 | KF442228 |
| Lh7        | F: AACATTCCACGCTGCTCA R: GGTACCGGCTGCTTAAATA | 60°C | (ATAG)_{10} | 185–189 | 2 | KF442229 |
| Lh9        | F: GCATGGGGAGCGCTTACAA R: GATTCGACCGTCTCAATA | 60°C | (AGAT)_{10} | 172–248 | 13 | KF442230 |
| Lh12       | F: CTCATACAGCCTGCTTGG R: GGTGACGCTCTTGTGCTAA | 60°C | (AGAT)_{19} | 164–176 | 4 | KF442231 |
| Lh13       | F: GTCGCCAGCGGCTGTAT R: CCTCCTGCAGTCCACTCA | 60°C | (AATC)_{16} | 188–208 | 5 | KF442232 |
| Lh14       | F: GACACATCTCTACGTCTGA R: AGGGATTAGACCACTCA | 60°C | (AATC)_{11} | 216–244 | 5 | KF442233 |
| Lh16       | F: TCAGCCTAGCCATTCACA R: TGATAGGAGGCGTCATATAAATAC | 60°C | (AATC)_{13} | 130–142 | 4 | KF442234 |
| Lh17       | F: GCACATGGGGAGCTCGA AAAA R: GACTTGAGCTTCCTAATAATAC | 60°C | (AGAT)_{10} | 175–215 | 4 | KF442235 |
| Lh18       | F: GCCACAGGAGTACCTCAAGT R: CAGTTGAGGAGGCTTACA | 60°C | (ACAT)_{11} | 137–157 | 5 | KF442236 |
| Lh19       | F: CAGGGGAGAGGCTTACA | 60°C | (AATC)_{10} | 191–215 | 6 | KF442237 |
| Lh44       | F: TTGGGAGACACACCGTATT R: CTCGCTCTAGGAGACACCTCA | 60°C | (AATC)_{15} | 231–259 | 6 | KF442238 |
| Us4        | F: CCGATCTCGAGGAGGACTA R: TGGGATGGTGTGCTAAAGGTG | 60°C | (ATATG) | 196–204 | 3 | KF442239 |
| Us9        | F: TGATGAGGAGGAGGACTA R: TGCAAGACAGACAGGCTGACA | 60°C | (ATGT)_{15} | 136–186 | 6 | KF442240 |

Figure 1. Male of the crested newt (Triturus cristatus), one of the target species for which microsatellite loci have been developed (photograph by B. Thiesmeier).
seems to be highly efficient when compared to pure Illumina-based sequencing as evidenced by comparing success rates of our study with the one of Peterman et al. (2013).

**Implications for the use of new microsatellite loci for endangered amphibian species**

The newly developed 11 (T. cristatus), 20 (C. asper), and 15 (L. helveticus) polymorphic tetra-nucleotide microsatellite loci will be a tremendous help in furthering our knowledge of the population biology of these locally endangered species, finally building a basis for improved conservation measures. For T. cristatus, for example, the set of 19 applicable microsatellite loci will facilitate a more detailed identification of population structure, dispersal, and migration rates across small geographic scales and will even allow for the genetic assignment of single individuals to populations with high credibility. In addition, estimates of effective population sizes of subpopulations or even populations from single ponds can now be identified with much higher resolution. The large number of newly developed applicable loci for C. asper sets the foundation for revealing the interesting population biology of this cryptic endemic mountain species, as well as that of its sister species C. arnoldi. In particular, new insights into dispersal propensity gained by genetic estimates will be important for elucidating population connectivity, the extent of single populations, the most common reproductive strategies, and how life-history traits relate to individual genotypes. Also, it can be tested whether the unexpected high genetic differentiation of C. asper populations based on AFLP markers (Milà et al. 2009) is corroborated by microsatellite loci.

Previous genetic studies in C. arnoldi suggested that eastern and western populations belonged to two evolutionary significant units (ESU’s; Valbuena-Ureña et al. 2013) and proposed the maintenance of a breeding program for individuals from both units separately. Further genetic studies using microsatellite loci markers to infer the genetic diversity of the species, the current gene flow among population and their possible isolation are urgently needed to evaluate the conservation status more precisely. With the ten C. asper microsatellite loci that successfully cross-amplified in C. arnoldi, we will be able to study the structure of different populations within the Montseny species range in much greater detail.

For L. helveticus, the new loci will be particularly useful for understanding the distribution and success of alternative phenotypes within the species range. Indeed, L. helveticus is one of the three European newt species in which facultative paedomorphosis is most regularly reported (Denoël 2007; Denoël et al. 2009). This process results in the retention of larval traits in adults in part of a population, while other individuals metamorphose into the terrestrial morph. Dimorphic populations are particularly common in southern France, where the highest rate of dimorphism is observed in an area that covers only 0.5% of the species range (Denoël 2007). The 23 microsatellite loci now available for this species will be useful for testing evolutionary hypotheses based on gene flow among paedomorphic and metamorphic individuals (see Denoël 2002).

**Conclusion**

The use of NGS (454 and Illumina sequencing) strongly facilitated the development of microsatellite loci. However, from most studies, it is unclear how effectively new microsatellite loci can be developed from the large number of sequencing reads obtained from NGS. Our comparative study on three distinct amphibian newt species demonstrates that, despite low overall success rates, the combination of enrichment protocols and NGS can result in considerably higher numbers of polymorphic tetra-nucleotide microsatellite loci. Our study draws a more realistic picture of the efficiency of microsatellite loci development in amphibian species and shows that 454-based microsatellite loci development is still competitive with Illumina-based approaches.

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Conflict of Interest

None declared.

References

Abdelkrim, J., B. C. Robertson, J.-A. L. Stanton, and N. J. Gemmell. 2009. Fast, cost-effective development of species-specific microsatellite markers by genomic sequencing. Biotechniques 46:185–191.

Beebee, T. C. J. 2005. Conservation genetics of amphibians. Heredity 95:423–427.

Carranza, S., and F. Amat. 2005. Taxonomy, biogeography and evolution of Euproctus (Amphibia: Salamandridae), with the resurrection of the genus Calotriton and the description of a new endemic species from the Iberian Peninsula. Zool. J. Linn. Soc. 145:555–582.

Carson, E. W., R. R. Beasley, K. L. Jones, S. L. Lance, M. D. L. Lozano-Vilano, L. Vela-Valladares, et al. 2013. Development of polymorphic microsatellite markers for the microendemic pupfishes Cyprinodon julimae and C. pachychale. Conserv. Genet. Resour. 5:853–858.

Castoe, T. A., A. W. Poole, W. Gu, A. P. J. de Koning, J. M. Daza, E. N. Smith, et al. 2010. Rapid identification of thousands of copperhead snake (Agkistrodon contortrix) microsatellite loci from modest amounts of 454 shotgun genome sequence. Mol. Ecol. Resour. 10:341–347.

Castoe, T. A., J. W. Streicher, J. M. Meik, M. J. Ingrasci, A. W. Poole, A. P. J. de Koning, et al. 2012a. Thousands of microsatellite loci from the venomous coralsnake Micrurus fulvius and variability of select loci across populations and related species. Mol. Ecol. Resour. 12:1105–1113.

Castoe, T. A., A. W. Poole, A. P. J. de Koning, K. L. Jones, and D. F. Tombback. 2012b. Rapid microsatellite identification from Illumina paired-end genomic sequencing in two birds and a snake. PLoS ONE 7: 1–10. doi:10.1371/ journal.pone.0030953.

Denoe, M. 2002. Paedomorphosis in the Alpine newt (Triturus alpestris): decoupling behavioural and morphological change. Behav. Ecol. Sociobiol. 52:394–399.

Denoe, M. 2007. Priority areas of intraspecific diversity: Larzac, a global hotspot for facultative paedomorphosis in amphibians. Anim. Conserv. 10:110–118.

Denoe, M. 2012. Newt decline in Western Europe: highlights from relative distribution changes within guilds. Biodivers. Conserv. 21:2887–2898.

Denoe, M., and G. F. Ficetola. 2007. Landscape level thresholds and newt conservation. Ecol. Appl. 17:302–309.

Denoe, M., G. F. Ficetola, R. Cirović, D. Radović, G. Đukić, M. L. Kalezić, et al. 2009. A multi-scale approach to facultative paedomorphosis of European newts in the Montenegro karst: distribution pattern, environmental variables and conservation. Biol. Conserv. 142:509–517.

Denoe, M., A. Perez, Y. Cornet, and G. F. Ficetola. 2013. Similar local and landscape processes affect both a common and a rare newt species. PLoS ONE 8: 1–11. doi: 10.1371/journal.pone.0062727.

Dobes, C. H., and S. Schefknect. 2012. Isolation and characterization of microsatellite loci for the Potentilla core group (Rosaceae) using 454 sequencing. Mol. Ecol. Resour. 12:726–739.

Dogaç, E., I. Kandemir, and V. Taskin. 2013. The genetic polymorphisms and colonization process of olive fly populations in Turkey. PLoS ONE 8:e56067. doi:10.1371/journal.pone.0056067.

Duque, T. Y., K. T. Scribner, P. S. Forsythe, J. A. Crossman, and E. A. Baker. 2013. Interannual variation in effective number of breeders and estimation of effective population size in long-lived iteroparous lake sturgeon (Acipenser fulvescens). Mol. Ecol. 22:1282–1294.

Fisher, M. C., B. R. Schmidt, K. Henle, D. S. Schmeller, J. Bosch, D. M. Aanensen, et al. 2012. RACE: Risk Assessment of Chytridiomycosis to European Amphibian Biodiversity. Froglog 101:45–47.

Gardner, M. G., A. J. Fitch, T. Bertozzi, and A. J. Lowe. 2011. Rise of the machines – recommendations for ecologists when using next generation sequencing for microsatellite development. Mol. Ecol. Resour. 11:1093–1101.

Geser, S., L. Kaiser, V. Zwahlen, and S. Ursenbacher. 2013. Development of polymorphic microsatellite loci markers for the Asp viper (Vipera aspis) using high-throughput sequencing and their use for other European vipers. Amphib-Reptil. 34:109–113.

Glenn, T. C., and N. A. Schable. 2005. Isolating microsatellite DNA loci. Methods Enzymol. 395:202–222.

Hauswaldt, J. S., J. Fussel, J. Guenther, and S. Steinfartz. 2008. Eight new tetranucleotide microsatellite loci for the agile frog (Rana dalmatina). Mol. Ecol. Resour. 8:1457–1459.

Hauswaldt, J. S., A. Polok, C. Angelini, and S. Steinfartz. 2012. First microsatellite loci forspectacled salamanders (Salamandra perspicillata and S. terdigitata) endemic to the Apennine peninsula. Conserv. Genet. Resour. 4:399–402.

Hendrix, R., J. S. Hauswaldt, M. Veith, and S. Steinfartz. 2010. Strong correlation between cross-amplification success and genetic distance across all members of ’True Salamanders’ (Amphibia: Salamandridae) revealed by Salamandra salamandra-specific microsatellite loci. Mol. Ecol. Resour. 10:1038–1047.

Hoban, S. M., O. E. Gaggiotti, the ConGRESS Consortium, and G. Bertorelle. 2013. Sample Planning Optimization Tool for conservation and population Genetics (SPOTG): a software for choosing the appropriate number of markers and samples. Methods Ecol. Evol. 4: 299–303. doi:10.1111/2041-210X.12025.

Jehle, R., and J. W. Arntzen. 2002. Microsatellite markers in amphibian conservation genetics. Herpetol. J. 12:1–9.

Jehle, R., T. Burke, and J. W. Arntzen. 2005. Delineating fine-scale genetic units in amphibians: probing the primacy of ponds. Conserv. Genet. 6:227–234.

Jehle, R., M. Szomicsny, J. B. W. Wolf, A. Whitlock, W. Hödl, and T. Burke. 2007. Genetic dissimilarity predicts paternity.
in the smooth newt (*Lissotriton vulgaris*). Conserv. Genet. 6:227–234.

Jehle, R., B. T., and J. Foster (2011) The crested newt. Laurenti-Verlag. Bielefeld, Pp. 152.

Johannet, A., D. Picard, T. W. J. Garner, D. A. Dawson, R. Morales-Hojas, R. Jehle, et al. 2009. Characterization of microsatellite loci in two closely related *Lissotriton* newt species. Conserv. Genet. 10:1903–1906.

Jones, A. G., E. M. Adams, and S. J. Arnold. 2002. Topping off a mechanism of first-male sperm precedence in a vertebrate. Proc. Natl Acad. Sci. USA 99:2078–2081.

Krupa, A. P., R. Jehle, D. A. Dawson, L. K. Gentle, M. Gibbs, J. W. Arntzen, et al. 2002. Microsatellite loci in the crested newt (*Triturus cristatus*) and their utility in other newt taxa. Conserv. Genet. 3:87–89.

Loyau, A., and D. S. Schmeller. 2012. Mixed reproductive strategies of the Common Moorhen on a micro-scale as revealed by genetic data. C.R. Biol. 335:673–679.

Marshall, T. C., J. Slate, L. E. B. Kruuk, and J. M. Pemberton. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Mol. Ecol. 7:639–655.

Mayer, C. 2007. Phosho: a tandem repeat search tool 2007. Available at http://www.rub.de/spezzoo/cm (accessed 6 September 2013).

Miľa, B., S. Carranza, O. Guillaume, and J. Clobert. 2009. Marked genetic structuring and extreme dispersal limitation in the Pyrenean brook newt *Calotriton asper* (Amphibia: Salamandridae) revealed by genome-wide AFLP but not mtDNA. Mol. Ecol. 19:108–120.

O’Byhím, J., J. P. Chong, S. L. Lance, K. L. Jones, and K. J. Roe. 2012. Development and characterization of sixteen microsatellite markers for the federally endangered species: *Leptodea leptodon* (Bivalvia: Unionidae) using paired-end Illumina shotgun sequencing. Conserv. Genet. Resour. 4:787–789.

Palo, J. U., D. Lesbarreres, D. S. Schmeller, C. R. Primmer, and J. Merilä. 2004. Microsatellite marker data suggests sex biased dispersal in the common frog *Rana temporaria*. Mol. Ecol. 13:2865–2869.

Peterman, W. E., L. R. Pauley, E. R. Brocato, E. C. Stuart, R. D. Semlitsch, and L. S. Eggert. 2013. Development and characterization of 22 microsatellite loci for the ringed salamander (*Ambystoma annulatum*) using paired-end Illumina shotgun sequencing. Conserv. Genet. Resour. doi:10.1007/s12686-013-9951-3.

Prunier, J., B. Kaufmann, O. Grolet, D. Picard, F. Pompanon, and P. Joly. 2012. Skin swabbing as a new efficient DNA sampling technique in amphibians, and 14 new microsatellite markers in the alpine newt (*Ichthyosaura alpestris*). Mol. Ecol. Resour. 12:524–531.

Rozen, S. and H. Skaltsky (2000) Primer3 on the WWW for general users and for biologist programmers. Pp. 365–386 in S. Krawetz, S. Misener, (eds). Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, NJ.

Schmeller, D. S., and J. Merilä. 2007. Demographic and genetic estimates of effective population size in the amphibian *Rana temporaria*. Conserv. Biol. 21:142–151.

Schmeller, D. S., A. Seitz, A. Crivelli, and M. Veith. 2005. Crossing species’ range borders: interspecies gene exchange mediated by hybridogenesis. Proc. Biol. Sci. 272:1625–1631.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nat. Biotechnol. 18:233–234.

Steinfartz, S. (2003) Molecular analysis of adaptive divergence: the impact of ecology on speciation in salamanders. Inaugural Dissertation, University of Cologne, Germany, Pp. 69.

Steinfartz, S., D. Küsters, and D. Tautz. 2004. Isolation and characterization of polymorphic tetranucleotide microsatellite loci in the Fire salamander *Salamandra salamandra* (Amphibia: Caudata). Mol. Ecol. Notes 4:626–628.

Steinfartz, S., K. Stemshorn, D. Küsters, and D. Tautz. 2006. Patterns of multiple paternity within and between annual reproduction cycles of the fire salamander (*Salamandra salamandra*) under natural conditions. J. Zool. 268:1–8.

Storfer, A., M. A. Murphy, S. F. Spear, R. Holderegger, and L. P. Waits. 2010. Landscape genetics: where are we now? Mol. Ecol. 19:3496–3514.

Valbuena-Ureña, E., F. Amat, and S. Carranza. 2013. Integrative phylogeography of *Calotriton* news (Amphibia, Salamandridae), with special remarks on the conservation of the endangered Montseny brook newt (*Calotriton arnoldi*). PLoS ONE 8: 1–12. doi: 10.1371/journal.pone.0062542.

Yang, T., S. Bao, R. Ford, T. Jia, J. Guan, Y. He, et al. 2012. High-throughput novel microsatellite marker of faba bean via next generation sequencing. BMC Genomics 13:602. doi:10.1186/1471-2164-13-602.

Zane, L., L. Bargelloni, and T. Patarnello. 2002. Strategies for microsatellite isolation: a review. Mol. Ecol. 11:1–16.

### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Data S1** Data Accessibility.

| Table S1 | Locus specific characteristics of two populations of *T. cristatus*. Population names, sample sizes (N), and observed (Ho) and expected (He) heterozygosities are provided. |
| Table S2 | Locus specific characteristics of up to 4 populations of *C. asper*. Population names, sample sizes (N), and observed (Ho) and expected (He) heterozygosities are provided. |
| Table S3 | Locus specific characteristics of 5 populations of *L. helveticus*. Population names, sample sizes (N), and observed (Ho) and expected (He) heterozygosities are provided. |
| Table S4 | Locus specific characteristics of two populations of *Calotriton arnoldi*. Population codes, sample sizes (N), and observed (Ho) and expected (He) heterozygosities are provided. |