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RESEARCH NOTE

An improved microsatellite panel to assess genetic variability of the Italian smooth newt (*Lissotriton vulgaris meridionalis*)

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Abstract. Habitat loss and fragmentation are threatening amphibians by increasing population isolation. However, artificial waterbodies created for livestock may contrast this phenomenon by providing surrogate habitats for amphibians. Here, we performed a genetic study on an amphibian species, *Lissotriton vulgaris meridionalis*, in a rural area in central Italy where natural wetlands are disappearing and drinking troughs for cattle watering are widespread. Specifically, we tested a panel of microsatellite markers to identify a suitable tool for addressing conservation genetic issues of this species that is undergoing severe local decline. Twelve of the 20 tested loci produced reliable amplifications and were polymorphic. Three distinct units with a low level of gene flow were distinguished and the population genetic structuring overlapped with geographic distribution. Such loci will be useful to assess the genetic diversity of the species across multiscale levels for its management and conservation.

Keywords. conservation genetics; drinking troughs; amphibians; population structure.

Introduction

In the past decades, amphibians have been undergoing a major global decline because of pollution, pathogens, exotic species, UV radiation, habitat destruction and climatic changes (Alford and Richards 1999). Habitat loss and fragmentation, consequent to agricultural intensification, have posed serious threats to many species by increasing population isolation (Beebee 2005). Definitely, intrinsic amphibian features such as metapopulation structure (Alford and Richards 1999), low dispersal abilities and strong site fidelity (Squire and Newman 2002) make them particularly vulnerable by limiting interpopulation exchanges and structuring populations in distinct genetic units despite geographic proximity (Jehle and Arntzen 2002). The study of amphibian population dynamics and connectivity, and the identification of demes vulnerable to genetic threats are therefore a priority from a conservation perspective, especially at a small geographic scale (Semlitsch 2000; Jehle and Arntzen 2002). In this context, microsatellite markers represent suitable genetic tools to identify populations with reduced genetic diversity, infer population structure, estimate effective population size, determine levels of migration and gene flow among populations, and investigate the effects of barriers and other landscape features on populations (Jehle and Arntzen 2002, Beebee 2005).

The smooth newt, *Lissotriton vulgaris* (Linnaeus 1758) is abundant and widely distributed in Europe (Razzetti and Bernini 2006), and is an ideal candidate for investigating regional population connectivity since it (i) has limited dispersal capabilities, (ii) exhibits high breeding site fidelity, and (iii) is a highly deme-structured species (Griffiths 1996; Roth and Jehle 2016). The smooth newt is not protected by Habitat Directive, although local populations seem to be undergoing severe declines, especially in Italy (ssp. *meridionalis*), following habitat destruction and fragmentation (Razzetti and Bernini 2006).

The aim of the present study was to test a panel of 20 microsatellite markers (previously developed and tested for cross-species amplification in other *Lissotriton* species and subspecies) on the Italian smooth newt, *L. v. meridionalis*

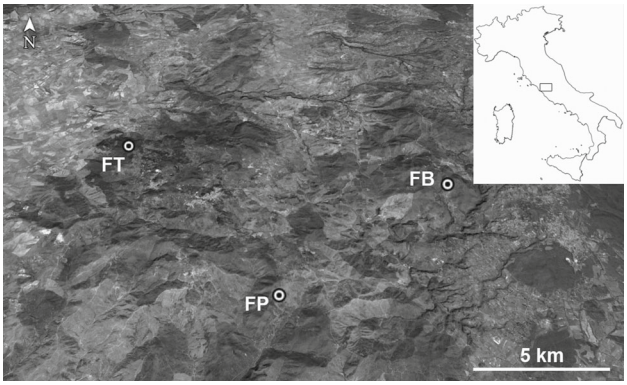

Figure 1. Satellite map of the study area with localities for the three sampled *L. v. meridionalis* populations inhabiting drinking trough FB, Bandita; FP, Pianoro; FT, TorCimina.

(Boulenger 1882), to identify a suitable tool for addressing conservation genetic issues of this potentially threatened species. Specifically, we aimed to evaluate the usefulness of this panel in assessing population structure and genetic variability at a local spatial scale.

Materials and methods

Field work was carried out during 2015 in a Special Protection Area (SPA, IT6030005) in Latium region (central Italy), where natural wetlands are disappearing and being no longer available for amphibian reproduction. However, drinking troughs constructed for free-ranging cattle watering are particularly widespread in the study area and may indirectly provide surrogate breeding habitats for amphibians. We collected tissue samples by tail clipping from 57 adult Italian smooth newts from three demes inhabiting drinking troughs 10–15 km far from each other (FB, Bandita; FP, Pianoro; FT, TorCimina. Distances (km): FP–FT = 11.7; FB–FP = 10.1; FT–FB = 15.7; figure 1). Tail-clips were stored in 95% ethanol until analysis.

Twenty published microsatellite loci were selected and tested (table 1). The forward primers were labelled with a fluorescent dye (Applied Biosystems, Foster City, USA). Newt tail-tips were digested using a proteinase K solution (56°C, overnight). Total genomic DNA was extracted using DNeasy blood and tissue kit (Qiagen, Hilden, Germany). Simplex PCRs were performed to test each of the 20 primer pairs. Among these, 12 produced reliable amplification with polymorphic results, whereas the remaining primers were discarded because of no amplification, low signal, or many aspecific signals (table 1). To optimize time and cost of our analyses, we ran three multiplex PCRs (M1, M2, M3; table 1) using Qiagen Multiplex PCR kit in 8 μL mix solution composed of 3.50 μL HotStarTaq-Master mix, 0.70 μL Qsol, 1 μL DNA and 0.20 μL each primer (10 μM) were brought to volume with H_2O. Amplification conditions for all loci were carried out, setting an initial denaturing at 95°C for 15 min, 40 cycles of denaturing at 94°C for 30 s, annealing temperature of 55°C for 1.30 min and 72°C extension for 1 min, and a final 10 min extension at 72°C. Two replicates for each sample at each locus were performed. PCR products were electrophoresed with internal size standard (GeneScan 1200 LIZ, Life Technologies, Carlsbad, USA) in an ABI3130XL sequencer and allele sizes were scored using the software GeneMapperv.4.0 (Life Technologies).

We checked newt genotypes for the presence of null alleles (Micro-Checker, Oosterhout *et al.* 2004), Hardy–Weinberg equilibrium within populations (Genepop ver. 4.2, Rousset 2008), and estimated average number of alleles per locus, allele frequencies, expected and observed heterozygosities, genetic distances and eligibility tests through principal coordinate analysis (PCoA) (Genalex ver. 6.5, Peakall and Smouse 2006, 2012). Then, we inferred population genetic structure and distinguished the clusters of populations by means of Bayesian procedures (Structure ver. 2.3.4, Pritchard *et al.* 2000). For evaluation of optimum number of populations (K), a simulation was coordinated using parameters K(1–3) with a random start for each K value and five independent runs (200,000 iteration following a burn-in period of 20,000), and assuming ΔK value, which takes into account the shape of the log likelihood curve (Evanno *et al.* 2005).

Results and discussion

Twelve of 20 tested microsatellite loci produced reliable amplifications. All loci were polymorphic except LVG-388 in FB and FP populations, and LVG-210 and Lm_013 in FT population. A similar low-level of successful cross-species amplification was documented in other *Lissotriton* species (Johanet *et al.* 2009; Nadachowska *et al.* 2010). This was not surprising since close related amphibian taxa (i.e., congeneric species and subspecies) often showed a cross-species amplification success rate lower than expected (Primmer and Merilä 2002). This is probably due to the intrinsic characteristics of urodelan genome (i.e., large-sized and complex) resulting in an amplification success that decreases significantly with its increasing size (Garner 2002). Further, microsatellites are usually found in noncoding regions with high substitution rates positively correlated with genetic divergence time (Primmer and Merilä 2002). Thus, the detected low cross-amplification success may also be explained by the fact that *Triturus* genus is no longer considered monophyletic and *Triturus* species diverged long time ago (i.e., more than 60 Mya; Steinfartz *et al.* 2007).

The number of alleles per locus ranged from two (LVG-388) to 18 (Lm_488 and Lm_749) and the expected heterozygosity from 0.046 (LVG-388) to 0.810 (Lm_521) (table 1). After Bonferroni correction, the observed and expected heterozygosities did not differ significantly

Table 1. Twenty published microsatellite loci, selected and tested on collected newt samples.

Locus	Fluorescent dye	Sequence (5′–3′)	Locus size range (bp)	N_a	H_o	H_e	Multiplex
Lm_013*	HEX	F: CTTGGTTCCCAGTGAGGAGA R: GCAAGCCATCCCAAAGTAAG	156–164	3	0.201	0.259	M3
Lm_488*	PET	F: CAGGCAGGGTATTTGCGTAG R: GGTCATTTCCACAACAAGCTC	236–522	18	0.864	0.783	M2
Lm_521*	FAM	F: CATACGGGCACTGAGGTGAT R: GCACAGACATTGATGGCAAA	236–320	13	0.797	0.810	M1
Lm_528*	NED	F: CTGGCTTGAAATGCCTTCAT R: AGGGCAGGGCTATACGTCTT	Many aspecific signals				
Lm_632*	FAM	F: CAGAGCAATTTCTAGGCAAGG R: GGCGCTATATCAAACTGCAA	176–244	14	0.780	0.680	M2
Lm_749*	PET	F: CCATGGTGGTAGAATAAATGGAA R: AAGACCATTCTTTCTGAGGTATCC	214–542	18	0.802	0.738	M3
LVG-210/EU568357#	HEX	F: ATGAGCCAACACCATGTCTG R: TGATGTCCTTGGGATCCTTG	222–228	4	0.190	0.156	M1
LVG-250/EU568353#	HEX	F: GCTTGGGAGAGCCCTATCTT R: CCAATAGTTTAATCTCTGGCAAATG	Many aspecific signals				
LVG-267/EU568359#	NED	F: CTTGTCTTTAAGGGCCCAAGT R: TCTGCACAACAACACAAGACC	Many aspecific signals				
LVG-303/EU568355#	FAM	F: AGTAGTGATCAGCGGCACAGT R: CGCTAGGAGGCAAGATCCTAT	Low signal				
LVG-388/EU568352#	PET	F: GTGGGTGGTAGGCCAGAATAC R: CCCATGAACAACCCATTAGG	157–159	2	0.050	0.046	M1
LVG-398/EU568356#	NED	F: ACCAGATATGTTCCGCTTCCT R: TTGCAAATAAAATAACGCTACCC	126–144	6	0.649	0.649	M3
LVG-449/EU568358#	HEX	F: AGGTCAGATGGGTTTCCTTGT R: GATCCGTAGTCAGACGCGTTA	Low signal				
LVG-542/EU568354#	HEX	F: ACAACACAGCCAGATTTCCAA R: TACAATGATCCTTTCGCCTTG	139–151	5	0.537	0.481	M1
Th09+	PET	F: CGACACTCGCAAATCAATC R: ATTATTATTTCCACACACGTAAACAT	137–177	10	0.640	0.735	M2
Th14+	HEX	F: CCCACTGGGAATCTGAGAAA R: TGCCATATTGTTGAGGACCA	No amplification				
ThCa14+	HEX	F: ACCTGCTGACAGTGCAAATG R: CGATTGATCATGGGACACGTA	No amplification				
Tv12+	FAM	F: AACCGGGTACACCTACATCA R: CTCACCTTTGTAATGGCCTCT	Many aspecific signals				
Tv3Ca9+	NED	F: AAATAACTTGTGATTGGGTCATTT R: TGCATATATACTGTATGTTTACTGCAA	79–111	3	0.532	0.504	M2
Tv3Ca19+	HEX	F: CCTCCACGAGGTTACTGCAC R: GACCATTTCAGACACTCATTACG	62–100	6	0.391	0.616	M3

For each locus shown: fluorescent dye used; microsatellite sequence; size range; N_a, allele number; H_o, observed heterozygosity; H_e, expected heterozygosity; multiplex PCRs performed.
*_L. montandoni_, Nadachowska *et al.* (2010).
#_L. vulgaris graecus_, Sotiropoulos *et al.* (2009).
+_L. helveticus_ and _L. vulgaris_, Johanet *et al.* (2009).

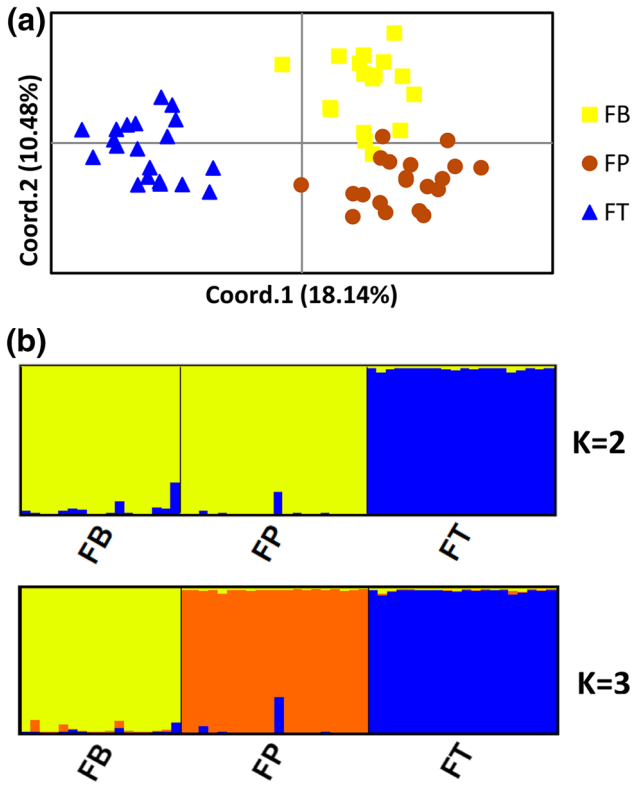

Figure 2. (a) PCoA of 57 smooth newt samples. (b) Bayesian clustering results regarding three smooth newt populations inhabiting drinking trough (FB, Bandita; FP, Pianoro; FT, TorCimina); (K = 1–3, pop-info = 0).

among the loci. Overall, the average number of alleles ranged from 4.333 (FP) to 5.667 (FB) and the expected heterozygosity from 0.465 (FT) to 0.600 (FB) (table 2). The estimated deviations from HWE were not significant for all loci. The three populations exhibited good levels of genetic variability for total values of average allele number (4.806), expected heterozygosity (0.538) and percentage of polymorphic loci (88.89%) (table 2). The detected genetic diversity suggests that the study populations may face a low risk of extinction by readily adapting to changing environmental conditions (Reed and Frankham 2003).

Both PCoA and Bayesian analyses distinguished three distinct units, differentiated and isolated from each other with a low level of gene flow (figure 2, a&b). Such population genetic structuring overlapped with geographic distribution with a highest differentiation among the farther demes. As established from Kdistribution, the graphical method detected the highest value of ΔK at $K = 3$ indicating optimum number of clusters (figure 2b), with a first split between FB–FP and FT (K= 2, figure 2b). Clustering of the populations was performed on the basis of genetic similarity among the groups irrespective of geographical locations of sampling. Differentiation between FB and FP (F_{ST}= 0.081) was low compared to what was estimated for FT. Indeed, FT was more geographically and genetically

Table 2. Estimates of population genetic diversity at 12 microsatellites.

Pop	N	%P	N_a	P_a	H_e	H_o	HWE
FB	17	91.67	5.667 (0.899)	2.000 (0.685)	0.600 (0.073)	0.581 (0.084)	0.002
FP	20	91.67	4.333 (0.595)	1.333 (0.355)	0.549 (0.076)	0.556 (0.084)	0.154
FT	20	83.33	4.417 (0.763)	1.167 (0.458)	0.465 (0.089)	0.472 (0.102)	0.192
Total	57	88.89	4.806 (0.440)	–	0.538 (0.046)	0.536 (0.051)	0.003

N, sample size; %P, percentage of polymorphic loci; N_a, allele number; P_a, private alleles; H_e, expected heterozygosity; H_o, observed heterozygosity; HWE, Hardy–Weinberg equilibrium; in brackets, standard error values.

isolated from the others by showing the highest pairwise F_{ST} values (0.129 with FB and 0.132 with FP). Amphibian populations often exhibit a high degree of spatial structure, particularly when interpopulation distances exceed several kilometres (Shaffer *et al.* 2000). In this case, the population differentiation positively correlated with the distance among breeding sites. However, the observed pattern could likely to be influenced also by landscape features (i.e., barriers and corridors) that facilitate/impede newt dispersal.

The set of markers, developed for other *Lissotriton* species–subspecies and optimized in the present study, represented a reliable tool for population genetic analyses in the ssp. *L. v. meridionalis*. Such loci had a good resolution even at a fairly fine-scale and will help to asses Italian smooth newt genetic diversity and understand its genetic structure across multiscale levels, from metapopulations to full geographical range. These markers will be helpful in planning strategies for effective management and conservation of the species.

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References

Alford R. A. and Richards S. J. 1999 Global amphibian declines: a problem in applied ecology. *Annu. Rev. Ecol. Syst.* **30**, 133–165.

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

Evanno G., Regnaut S. and Goudet J. 2005 Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol. Ecol.* **14**, 2611–2620.

Garner T. W. J. 2002 Genome size and microsatellites: the effect of nuclear size on amplification potential. *Genome* **45**, 212–215.

Griffiths R. A. 1996 *Newts and salamanders of Europe*. Poyser/Academic Press, London and San Diego.

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

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

Nadachowska K., Flis I. and Babik W. 2010 Characterization of microsatellite loci in the Carpathian newt (*Lissotriton montandoni*). *Herpetol. J.* **20**, 107–110.

Peakall R. and Smouse P. E. 2006 GenAlEx 6: genetic analysis in excel. Population genetic software for teaching and research. *Mol. Ecol. Notes* **6**, 288–295.

Peakall R. and Smouse P. E. 2012 GenAlEx 6.5: genetic analysis in excel. Population genetic software for teaching and research—an update. *Bioinformatics* **28**, 2537–2539.

Primmer C. R. and Merilä J. 2002 A low rate of cross-species microsatellite amplification success in Ranid frogs. *Conserv. Genet.* **3**, 445–449.

Pritchard J. K., Stephens M. and Donnelly P. J. 2000 Inference of population structure using multilocus genotype data. *Genetics* **155**, 945–959.

Razzetti E. and Bernini F. 2006 *Triturus vulgaris*. In *Atlas of Italian amphibians and reptiles societas Herpetologica Italica* (ed. R. Sindaco, G. Doria, E. Razzetti and F. Bernini), pp 230–235. Edizioni Polistampa, Firenze.

Reed D. H. and Frankham R. 2003 Correlation between fitness and genetic diversity. *Conserv. Biol.* **17**, 230–237.

Roth S. and Jehle R. 2016 High genetic diversity of common toad (*Bufo bufo*) populations under strong natural fragmentation on a Northern archipelago. *Ecol. Evol.* **6**, 1626–1636.

Rousset F. 2008 GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Mol. Ecol. Resour.* **8**, 103–106.

Semlitsch R. D. 2000 Principles for management of aquatic-breeding amphibians. *J. Wildlife Manage.* **64**, 615–631.

Shaffer H. B., Fellers G. M., Magee A. and Voss S. R. 2000 The genetics of amphibian declines: population substructure and molecular differentiation in the Yosemite Toad, *Bufo canorus* (Anura, Bufonidae) based on single-strand conformation polymorphism analysis (SSCP) and mitochondrial DNA sequence data. *Mol. Ecol.* **9**, 245–257.

Sotiropoulos K., Eleftherakos K., Tsaparis D., Kasapidis P., Magoulas A. and Legakis A. 2009 New polymorphic microsatellite loci for the Greek smooth newt, *Lissotriton vulgaris graecus*, and their utility in the nominotypical subspecies. *Mol. Ecol. Resour.* **9**, 292–295.

Squire T. and Newman R. A. 2002 Fine-scale population structure in the wood frog (*Rana sylvatica*) in a northern woodland. *Herpetologica* **58**, 119–130.

Steinfartz S., Vicario S., Arntzen J. W. and Caccone A. 2007 A Bayesian approach on molecules and behaviour: reconsidering evolutionary patterns in *Triturus* newts (Amphibia: Salamandridae). *J. Exp. Zool. B. Mol. Dev. Evol.* **308**, 139–162.

Van Oosterhout C., Hutchinson W. F., Wills D. P. and Shipley P. 2004 MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* **4**, 535–538.

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