SALAMANDRA **55**(3) | 191–198 | 15 August 2019 | ISSN 0036–3375

Development of new microsatellite markers for the Green Toad, *Bufotes viridis,* to assess population structure at its northwestern range boundary in Germany

Miguel Vences[1], R. G. Bina Perl[2], Katinka Giesen[3], Rieke Schluckebier[3], Klaus Simon[4], Elmar Schmidt[4], Sebastian Steinfartz[1] & Thomas Ziegler[3,5]

[1)] Zoological Institute, Braunschweig University of Technology, Mendelssohnstr. 4, 38106 Braunschweig, Germany
[2)] Faculty of Marine Sciences, Ruppin Academic Center, Mikhmoret 40297, Israel
[3)] Institute of Zoology, University of Cologne, Zülpicher Str. 47b, 50674 Köln, Germany
[4)] NABU-Naturschutzstation Leverkusen-Köln, Friedrich-Ebert-Str. 49, 50996 Köln
[5)] Cologne Zoo, Riehler Str. 173, 50735 Köln, Germany

Corresponding author: Miguel Vences, e-mail: m.vences@tu-braunschweig.de

Manuscript received: 23 March 2019
Accepted: 10 May 2019 by Stefan Lötters

Abstract. The Green Toad (*Bufotes viridis*) in Germany is mostly confined to secondary habitats and is experiencing severe population declines especially at its northwestern range boundary in the city of Cologne. As a basis for conservation management of this species, viz. to investigate the status of the population structure, we used a library of over 15,000 short tandem repeats to establish a new set of 12 microsatellite markers for this species. We applied these markers to *B. viridis* individuals from six sampling sites in Cologne, and included outgroup samples from sites in northern and central Germany. The results suggest the sampled Cologne toads form four distinct populations, without an obvious distinction between sites east and west of the river Rhine. Only the green toads of three neighbouring sampling sites clustered as one population, supporting the initial hypothesis that individuals from proximate sampling sites are genetically more similar than those from more isolated sampling sites. However, mitochondrial DNA sequences revealed a haplotype east of the Rhine that was absent west of the river. Toads from the northernmost sampled population in Cologne "Ginsterpfad" were not genetically depauperate, indicating that the severe declines at this site did not lead to a loss of genetic variation and were not related to inbreeding. Some Ginsterpfad individuals were genetically highly distinct from others, which may be explained by human introduction. Despite the encountered population structure, the low number of private alleles in the populations west of the Rhine, and the uniformity in mitochondrial haplotypes with the exception of one deviant allele east of the Rhine, lead us to recommend a pragmatic approach for conservation management. We suggest to consider all Cologne Green toads as belonging to a single management unit, but propose that, wherever possible, populations east and west of the Rhine should not be mixed in reintroduction measures.

Key words. Amphibia, Anura, microsatellites, conservation genetics, Cologne, North Rhine-Westphalia.

Introduction

The Green Toads, *Bufotes,* form a Palearctic genus of anurans with a clear centre of diversity in Asia. They represent a unique evolutionary and genetic model system due to their astonishing variation in ploidy levels (Stöck et al. 2002, 2006, 2009). In central Europe, two Green Toad lineages have been identified by the pioneering study of Stöck et al. (2006) but there is still controversy whether they should be recognized as distinct species *Bufotes variabilis* and *B. viridis* (Stöck et al. 2006, 2008, 2016 vs. Speybroeck et al. 2016). All German populations of Green Toads are classically considered to belong to the widespread species *Bufotes viridis* (Günther & Podloucky 1996), which reaches its northwestern range boundary in the German state of North Rhine-Westphalia, and more specifically, around the city of Cologne (Glaw & Vences 1989, Vences et al. 2003a, 2011).

Primary habitats of the Green Toad in this region can be assumed to have been located along the floodplains of the river Rhine, which we hypothesize have served as a route for postglacial colonisation of this region. In historical times, the species furthermore benefitted from a plethora of small excavations and gravel pits that provided an ideal, dense and dynamic network of open, unforested habitats in early stages of succession as well as temporary lentic wa-

ter bodies needed by this pioneer species (Vences et al. 2003a). In more recent times, increased regulation of gravel and sand exploitation has led to a reduction in number and increase in size of gravel pits in the region, and many of the now more intensely exploited pits are of limited value for the Green Toad (Glaw & Vences 1989; Vences et al. 2003a,b, 2011), which consequently has experienced important declines. Specifically in the city of Cologne, Glaw & Vences (1989, 1991) observed *Bufotes viridis* at 22 sites; about ten years later, the species had disappeared at seven of these sites, while only three newly colonised sites could be identified (Vences et al. 2003a, 2011). Currently, the situation has become even more dramatic, with the Green Toad in severe decline or extinct at various additional locations in Cologne (Schmidt & Simon 2017).

For an effective and scientifically informed conservation management of Green Toads in Cologne and elsewhere, genetic data are in some cases necessary. Despite the advent of population genomics with numerous powerful applications relying on next-generation sequencing, microsatellites or short-tandem repeats are still the work horse for small-scale conservation genetic studies (Selkoe & Toonen 2006). Such markers have been used to understand the genetics of contact zones among Green Toad species (e.g, Dufresnes et al. 2014), but have not been specifically developed for, and applied to, *B. viridis*. In this study, we developed an extensive new microsatellite library specifically for this taxon, established 12 microsatellite loci from this library, and applied it to investigate population structure among the Cologne populations of this species. As a test case to prove the usefulness of this new molecular resource, we used it in concert with DNA sequences of two mitochondrial genes to investigate the conservation genetics of Green Toad populations from Cologne: (1) Given the sparse genetic sampling of Green Toads in western Germany in previous studies (e.g., Stöck et al. 2006) and presumably high incidence of human translocation, it was first necessary to ascertain that the target populations all belong to the same genetic lineage rather than to the *variabilis* lineage or other (introduced) Green Toad species. (2) Secondly, the degree of genetic differentiation and isolation needed to be assessed to understand whether gene flow among populations occurs and whether some populations were sufficiently distinct to qualify as separate management units for conservation (Moritz 1994). (3) And thirdly, given the population crashes observed for some populations, especially at a site called "Ginsterpfad", it appeared important to understand whether these populations might be genetically depauperate to a degree that possible inbreeding depression could be partly responsible for the declines.

Material and methods

Tail clips from Green Toad larvae from two sampling sites in Germany, Cologne (North Rhine-Westphalia) and Schöningen (Lower Saxony), were used for development of a microsatellite library at the Sequencing Genotyping Facility, Cornell Life Sciences Core Laboratory Center (CLC), U.S.A. In brief, genomic DNA was extracted from the tissue samples with a Qiagen Blood and Tissue Kit (Qiagen, Hilden, Germany), digested in three separate reactions with the restriction enzymes AluI, RsaI, and Hpy166II, and combined in equal amounts after heat inactivation of the restriction enzymes. The blunt ends were adenylated (+A) with Klenow (exo-) and dATP, and after heat inactivation of the Klenow (exo-), the reactions were supplemented with ATP to 1 mM and an Illumina Y-adaptor was ligated with T4 DNA ligase. The fragments were enriched for microsatellites by hybridisation to and magnetic capture of biotinylated repeat probes (representing two unique dimers, five unique trimers, seven unique tetramers and two unique pentamers), amplified and barcoded by PCR, and sequenced on an Illumina MiSeq instrument (2 × 250 bp paired reads). The raw reads were assembled in SeqMan NGen (version 11). The assembly was scanned for microsatellite loci and automatically designed primer pairs with the program msatcommander 1.0.8_beta (for Mac OSX). A library was constructed with minimum consecutive perfect repeat lengths of at least six (12 bp) for any dimer and at least five for any trimer, tetramer, or pentamer and PCR product size of 150–450 bp. The full library is available as supplementary information (Supplementary Table S1) and from Figshare under DOI 10.6084/m9.figshare.8378849. Out of this library, we chose 12 loci based on following criteria (Perl et al. 2018): (i) tetrameric, (ii) repeat motif between 10 and 15, (iii) less than 1000 reads, as deep coverage could indicate multiple copies and (iv) GC content of 50 (Table 1). We tested the selected loci for successful amplification and for yielding unambiguously scorable and polymorphic PCR products.

We sampled 130 green toads from six sites in Cologne from 9 April 2016 to 20 June 2016, and 10 May 2017 to 11 August 2017, three individuals from Schöningen, and two individuals from a third locality, the island Fehmarn in northern Germany (Supplementary Table S2) for further validation of our results, given that these geographically distant sites were expected to show substantial genetic differentiation from each other and from the Cologne populations. For sampling locations, and geographical coordinates, see Table 2. We took either saliva swabs or tiny excisions of the toe webbing as tissue samples, and extracted DNA from these samples using a standard salt extraction protocol (Bruford et al. 1992).

We amplified fragments of the mitochondrial genes for 16S rRNA (16S) and cytochrome b (cob) using primers Cytb-a and Cytb-c (Bossuyt & Milinkovich 2000) and 16Sar-L and 16Sbr-H (Palumbi et al. 1991) following standard PCR protocols, and sequenced them on an ABI 3130xl capillary sequencer. Sequences were quality-checked, trimmed, and compared in Codon Code Aligner (CodonCode corp.). All newly determined sequences were submitted to Genbank (accession numbers MK882527–MK882585 and MK890047–MK890098). Microsatellites were amplified following the nested protocol of Schuelke (2000), but rather than a M13 sequence we used the Illumina se-

Table 1. List of forward (Fwd) and reverse (Rev) primers for 12 newly established microsatellite markers for *Bufotes viridis*. Repeat counts are from the initial library; numbers and length ranges of alleles, as well as % missing data, refer to the entire set of 84 samples. Length range (inferred bp) include primers and linker. * Locus Bvir16861 was suggestive of a null allele due to an excess of homozygotes.

Marker	5'-3' primer sequence	Repeat	Repeat count	N alleles	Length range	% Missing data
Bvir15979	Fwd: GAAATGAGTGGTGGTGAAGGAC Rev: GTTCCTGCTTCTTTCCTGAACC	AGAT	11	10	175–310	4.8
Bvir23976	Fwd: GCCTTTACTGATACTGGAGTGC Rev: CTACCTGGGAGTTCATAATGGG	AGAT	13	6	180–200	6.0
Bvir19382	Fwd: ACCATAACCAACAGACAGGAGG Rev: CTCCCTTTCTCTGCATAAAGGG	AGAT	10	12	189–241	6.0
Bvir11064	Fwd: CCATTCCTTTCTTGCTGGAGAC Rev: TTTCCAGCTCTCTCTATGCAGG	AGAT	11	4	200–212	2.4
Bvir10561	Fwd: ATGATAGTGCCCTCTTCTGGAC Rev: GCCACCATATACAGTACTGCAG	AGAT	13	8	205–261	0.0
Bvir5761	Fwd: CAGCTTTATACCTTGGACCAGC Rev: CCTCATTGTGTACGGCTGAAAG	AGAT	10	7	214–238	1.2
Bvir3372	Fwd: CTGAAGCGTTGCAACACCTATG Rev: AAGAAGAATCATCAGGGTCCGG	AGAT	14	7	277–305	2.4
Bvir11600	Fwd: TTCCCTTACGTCCTAACCAGTG Rev: CTTCCTGGAATTCTCACTCCAC	AGAT	13	8	226–268	19.0
Bvir3022	Fwd: ACAAAGGAAAGAGGTAGGGAGG Rev: GGGTGGTTTGTGGCAATATTCC	AAAG	11	11	231–357	0.0
Bvir16861*	Fwd: CACCTCTTCATAACTTTGGCGG Rev: CTTTCCTCCAAACCATTCCCAC	AGAT	14	7	391–412	2.4
Bvir29737	Fwd: AGTGTGACTGTATCTTCTGGCC Rev: TGTACATGGCAGAGGAGTGAAG	AGAT	13	13	151–211	20.2
Bvir13782	Fwd: CCAGTTATAAGAGGGTGTGCAC Rev: TAGAATTGTACACCCTGCTCCC	AGAT	14	10	432–464	0.0

Table 2. Geographical coordinates of localities (Lat, latitude; Long, longitude) sampled for *Bufotes viridis*, and summary statistics for the newly developed microsatellite markers. Values are given as sum, or as average ± standard deviation over all markers present in the respective population.

Location	East/West of Rhine	Lat	Long	Number of samples	Private/shared alleles	Average no alleles ± SD	Observed heterozygosity ± SD
Ginsterpfad	West	50.9851	6.9301	15	2/54	3.50 ± 1.38	0.5342 ± 0.1745
Immendorf	West	50.8516	6.9450	16	0/36	4.67 ± 1.92	0.6083 ± 0.2623
R 2.12	West	50.8449	6.9535	4	0/49	3.18 ± 0.98	0.7045 ± 0.2185
Kiesgrube Esser	West	50.8420	6.9304	8	1/41	4.08 ± 1.24	0.6354 ± 0.2290
Westhovener Aue	East	50.9037	7.0085	17	5/39	3.91 ± 1.04	0.4990 ± 0.1683
Porz-Wahn	East	50.8618	7.0774	19	4/51	4.58 ± 1.98	0.6540 ± 0.2751
Schöningen	East (Outgroup)	52.0836	10.5725	3	9/16	2.44 ± 0.73	0.4444 ± 0.2359
Fehmarn	East (Outgroup)	54.2811	11.0849	2	10/16	2.75 ± 0.87	1.0000 ± 0.0000

quencing primer sequence (ACACTCTTTCCCTACACGACGCTCTTCCGATCT) as linker, i.e., this sequence preceded all forward primers and was included as a FAM-labelled linker in the PCR. The amplification protocol consisted of 15 min of initial denaturation at 94°C, 30 cycles of 94°C (30 s), 60°C (45 s), 72°C (45 s), followed by 8 cycles of 94°C (30 s), 53°C (45 s), 72°C (45 s), and a final elongation step of 10 min at 72°C. We diluted PCR products once with 15 µl of RNase-free water, added 15 µl of Genescan 500–ROX size standard (Applied Biosystems) to 1 µl of each diluted product, and performed fragment analysis on an ABI 3130xl Genetic Analyzer. Alleles were called with GeneMapper® (SoftGenetics, State College, PA, U.S.A). We used MICRO-CHECKER version 2.2 (Van Oosterhout et al. 2004) to check for potential scoring errors, large allele dropout and the presence of null alleles. Hardy-Weinberg

equilibrium and linkage disequilibrium were tested in Arlequin (Excoffier et al. 2005) under Bonferroni correction (Rice 1989), and this program was also used to calculate pairwise F_{ST} values among all collection sites using the eight loci with missing data < 5%.

Population structure was analysed with STRUCTURE version 2.3.4 (Pritchard et al. 2000) under the assumption of an admixture model with correlated allele frequencies and without locprior. The number of clusters (K) was compared with 1 million Markov Chain Monte Carlo (MCMC) iterations and a burn-in of 100,000, repeating each assessment of K ten times. We selected the optimal number of clusters following the ΔK method by Evanno et al. (2005) using STRUCTURE HARVESTER (Earl & von Holdt 2012). We further used the Excel Microsatellite Toolkit (S.D.E. Park, 2008 – computer program and documentation distributed by the author) to calculate allelic diversity (shared and private alleles per population) as well as an individual allele-sharing matrix.

Results and Discussion

Mitochondrial differentiation of *Bufotes viridis* in Cologne

Mitochondrial DNA sequences for fragments of the 16S and cytochrome b genes were obtained for 58 and 62 specimens, respectively, covering all sites studied in Cologne (Fig. 1B; Supplementary Table S3). All sequences belonged to the *B. viridis* lineage and sequences were highly similar to each other: (i) in cytochrome b, the Schöningen samples differed from Cologne samples by one mutation, and (ii) in 16S, two individuals from Porz-Wahn and two specimens from Westhovener Aue had a haplotype differing by one mutation. Taken together, these data confirm that the studied samples belong to the *B. viridis* lineage, do not present major genetic differences and therefore can be analysed without restriction using the microsatellite markers. The

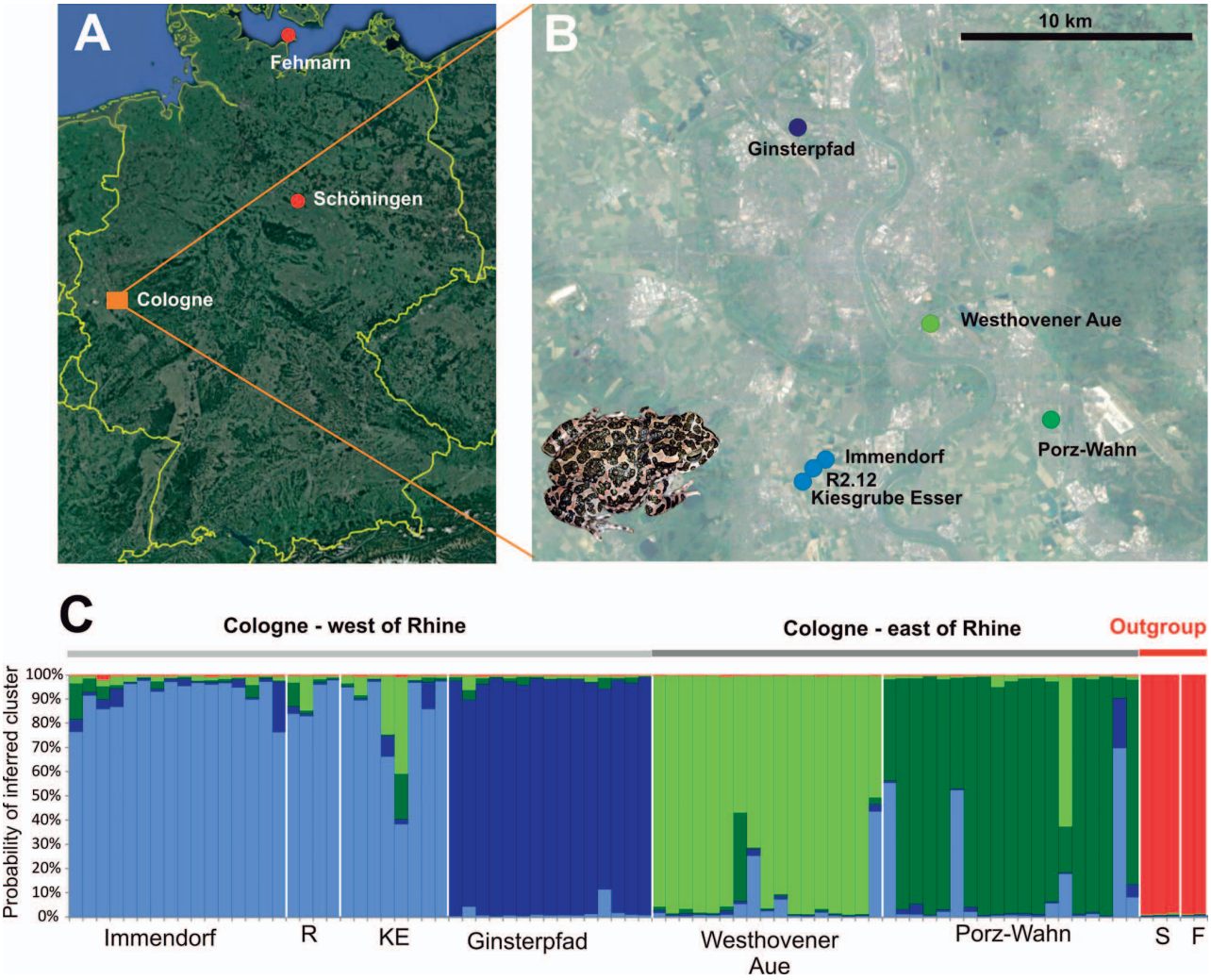

Figure 1. A–B) Maps showing sampling sites for individuals of *Bufotes viridis*, and C) barplot from an analysis with STRUCTURE with K=5 for 84 individuals. Each individual is represented by a single vertical line broken into K coloured segments, with height proportional to each of the K inferred clusters. Abbreviations: R, R2.12; KE, Kiesgrube Esser; S, Schöningen; F, Fehmarn.

Table 3. Pairwise F_{ST} values between populations of *Bufotes viridis* calculated from eight microsatellite loci with <5% missing data (after exclusion of loci Bvir 23976, Bvir 19382, Bvir11600, Bvir29737). All comparisons except those in italics were statistically significant.

	1	2	3	4	5	6	7	8
1 Immendorf	0.000							
2 R2.12	*0.014*	0.000						
3 Kiesgrube Esser	*0.024*	*0.000*	0.000					
4 Ginsterpfad	0.143	0.214	0.156	0.000				
5 Westhovener Aue	0.132	0.142	0.120	0.205	0.000			
6 Porz-Wahn	0.106	0.083	0.148	0.171	0.188	0.000		
7 Schöningen	0.359	0.395	0.369	0.368	0.423	0.357	0.000	
8 Fehmarn	0.358	*0.370*	0.362	0.372	0.450	0.330	*0.396*	0.000

distinct 16S haplotype occurring at the two sites east of the Rhine (Porz-Wahn and Westhovener Aue), although differing by a single mutation only, suggests a possible differentiation of these populations.

Characteristics of 12 new microsatellite markers for *Bufotes viridis*

After an initial screening of 20 loci from the newly sequenced library, we retained 12 loci for analysis (Table 1). The retained 12 loci all had tetranucleotid repeats (AGAT in 11 loci, AAAG in one locus), and all of them produced clear peaks without substantial stutter bands. We used a total of 84 samples from our target populations of *Bufotes viridis* for analysis. For these samples, number of alleles per locus was 4–13, missing data was 0–20%, and allele sizes (including primers and linker) ranged from a minimum of 151 bp in locus Bvir29737 to a maximum of 464 bp in locus Bvir13782 (Table 1).

We did not find scoring errors or large allele dropouts in our microsatellite data set, but one locus (Bvir16861) was suggestive of a null allele due to an excess of homozygotes. Nearly all loci at the majority of collecting locations were in the Hardy-Weinberg equilibrium. Specimens from the localities Ginsterpfad and Westhovener Aue each had one locus deviating from Hardy-Weinberg equilibrium, and locus Bvir11064 in R2.12 as well as locus Bvir3022 in Westhovener Aue were monomorphic. Linkage disequilibrium occurred in four out of the six sampling sites, but the linkage values differed from each other and thus no consistent linkage disequilibrium between any two loci was observed, i.e., the loci can be considered to be unlinked in the genome.

Genetic differentiation of *Bufotes viridis* in Cologne based on microsatellites

Our data set consisted of 4–19 individuals per site for six sampling sites in Cologne, and 2–3 individuals per site for the two geographically distant control sites (outgroups), Schöningen and Fehmarn (Figs 1A–B). Averaged over all loci, in Cologne we found 3.2–4.7 alleles and observed heterozygosities between 0.46–0.65 per site; total number of alleles per site was 36–54, of which up to 5 were exclusive to single sites (private alleles) (Table 2; see Supplementary Table S4 for details of expected and observed heterozygosities). F_{ST} values (calculated from a subset of eight loci with less than 5% missing data; Table 3) between the Cologne sites ranged between 0.014–0.214 and were statistically significant except for the three lowest ones between the three neighbouring sites, Immendorf, R2.12, and Kiesgrube Esser. As expected, the two geographically distant control sites (Schöningen and Fehmarn), despite the small sample sizes of 3 and 2 individuals, had a higher number of 9–10 private alleles (Table 2) and higher F_{ST} values of 0.36–0.45 to the Cologne populations and to each other (Table 3).

Analysis with STRUCTURE revealed a population count of K=5 or K=6 as favoured solutions based on highest likelihood values and lowest variation between runs (Supplementary Fig. S1). Because the two outgroup sites, Schöningen and Fehmarn, could not be adequately separated by this approach, probably due to low sample sizes, we chose a K=5 solution to represent the clustering of *B. viridis* individuals into populations (Fig. 1), which is also in accordance with the highest value for ΔK according to Evanno et al. (2005) (Supplementary Fig. S1). Alternative runs excluding locus Bvir16861 that was suggestive of a null allele (Supplementary Figure S2) or the two outgroup sites (not shown) resulted in very similar results. The analysis placed the three neighbouring sites (Immendorf, R2.12, and Kiesgrube Esser) into one population, and each of the remaining sites in Cologne (Ginsterpfad, Westhovener Aue, Porz-Wahn) into separate populations (Fig. 1C). This agrees with the comparatively low F_{ST} values between the three neighbouring sites, and the higher values between all other sites (Table 3). Overall, the STRUCTURE results suggest admixture, immigration and emigration among populations to be comparatively limited. Some individuals of Kiesgrube Esser had relatively high clustering probabilities with populations east of the Rhine, and some samples from Westhovener Aue and Porz-Wahn had relatively high clustering probabilities with populations west of the Rhine (especially one sample, MVTIS 4102). Interestingly, no substantial admixture was reconstructed among the two populations

west of the Rhine (Ginsterpfad and Immendorf/R.2.12/Kiesgrube Esser); and only one sample each of Westhovener Aue and Porz-Wahn, east of the Rhine, showed some indication of admixture among these two populations. The Rhine at Cologne has a width of over 200 m and therefore is without doubt a formidable barrier to dispersal of many terrestrial organisms. Although crossing such water barriers should, in principle, be possible for amphibians, multiple studies have shown that dispersal over rivers is at least limited in these organisms (e.g., Gascon et al. 2000; Gehring et al. 2012; Wollenberg Valero 2015; Fouquet et al. 2015). We therefore assume that natural dispersal over the Rhine is rare, and hypothesise that the possible genetic admixture between populations east and west of the river could be an indication of human translocations, either accidental (e.g., along with transport of construction materials) or deliberate. Alternatively, in the recent past (before the river was regulated), Green Toad populations may have occurred in wetlands along its edges and may have been transported across during floods.

An individual allele-sharing matrix (Fig. 2) confirmed the expected strong divergence of the two control groups, but also revealed up to four individuals in the Ginsterpfad population that shared surprisingly few alleles with individuals of all other Cologne sites, and at least one of them (MVTIS 4309) even shared only few alleles with other syntopic individuals. Yet, all individuals from the Ginsterpfad population came out homogeneous in the same cluster in the STRUCTURE analysis (Fig. 1). We interpret this pattern as an indication of possible genetic contribution of translocated individuals to the Ginsterpfad population; however from individuals originating from other, geographically distant populations not sampled herein.

Conservation genetics of *Bufotes viridis* populations in Cologne

The available evidence suggests *B. viridis* populations in Cologne are genetically not admixed yet characterised by occasional migration or human translocation of individuals. From a population genetic perspective, the rather distinct clusters revealed by STRUCTURE analysis, as well as the rather high F_{ST} values between most sites in Cologne, indicate that these do not behave as a single panmictic population and gene flow among them is restricted. Although *B. viridis* is a fast and formidable coloniser able to colonize new habitat over distances of up to 10 km (Geil 1962, Dalbeck et al. 1997) the urban landscape apparently acts as a strong barrier inhibiting regular gene flow, even for this pioneer species. Unsurprisingly, gene flow is not hampered among the neighbouring sites, Immendorf, R2.12 and Kiesgrube Esser, and these therefore cluster as a single population and have very low F_{ST} values.

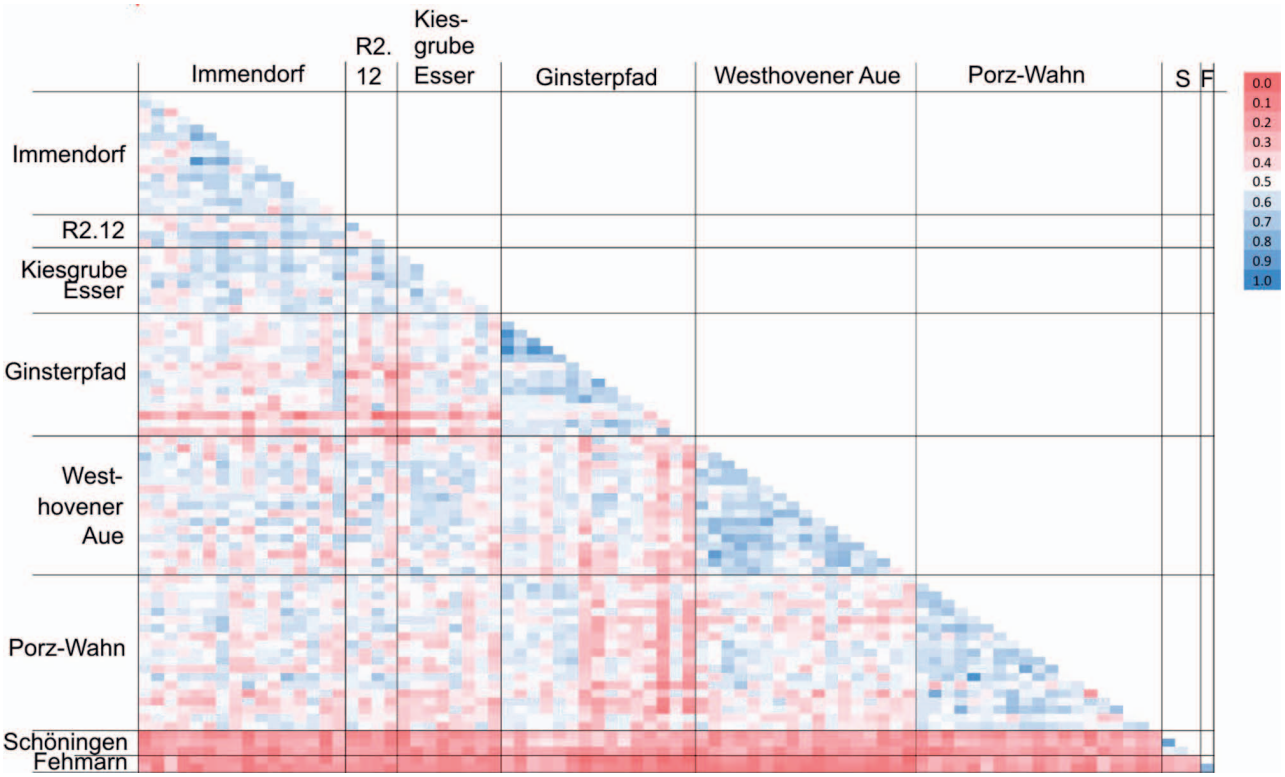

Figure 2. Individual allele-sharing matrix comparing individuals from all sampling sites (S, Schöningen; F, Fehmarn). Every cell represents the percentage of alleles shared by two individuals. Red colours indicate high genetic dissimilarity, blue colours high genetic similarity among individuals.

Despite this genetic isolation of the populations, at present there is no compelling evidence to consider them as separate management units from a conservation genetics perspective. The low number of private alleles per population and absence of mitochondrial differences suggest that their genetic divergence is recent – certainly post-glacial – and given their highly dynamic habitats it is very unlikely that any of the populations would have evolved local adaptations. For ongoing conservation management, e.g., human-aided recolonisation and translocation to newly created habitats, there seems to be no obvious need to keep these populations strictly separate, although it is, of course, always recommended to use individuals from geographically close populations to seed newly created habitats. However, as a cautionary measure, given that a different mitochondrial haplotype was found in the populations east of the Rhine, we recommend avoiding, as much as possible, mixing these populations with those west of the Rhine.

As a further aspect, no inbreeding depression due to reduced population size (Schmidt & Simon 2017) was found affecting any of the Cologne populations, considering the values of observed heterozygosity. Thus, other factors need to be explored to better understand the declines of these toads for instance at the Ginsterpfad population.

Conclusions and perspectives

In this study, we report on a somewhat limited number of 12 microsatellite markers, but the newly developed library will allow to easily select, test and establish many additional loci, if required. Although we did not perform cross-amplification tests, we expect many of these markers will also be applicable to other species of *Bufotes*. Besides providing information for local conservation management in Cologne, the novel molecular resources introduced here will therefore be of importance for future evolutionary and conservation research on *B. viridis,* and the entire, biologically intriguing genus *Bufotes*.

Acknowledgments

We are grateful to Steven M. Bogdanowicz (Cornell University) for his reliable service in microsatellite library development, to several friends and colleagues (Malou Raschewski, Maria Sachs, Oliver Töplitzer, Luke Schluckebier) for assistance during field work, to Matthias Stöck (Leibniz Institute of Freshwater Ecology and Inland Fisheries, Berlin) for valuable discussions on available microsatellite markers for *Bufotes*, to Burkhard Thiesmeier for providing samples from Fehmarn, to Anna Rauhaus (Cologne Zoo) for general logistic support, and to Gabriele Keunecke and Meike Kondermann for help in the laboratory. Special thanks go to Sabrina Henne, Miriam Labusch and Melina Wagler who performed part of the laboratory work and statistical analyses. Permits were granted by the Untere Naturschutzbehörde Cologne. Thanks to Theo Pagel (Cologne Zoo) for supporting the project, which was funded by intramural funds of Technische Universität Braunschweig and Cologne Zoo.

References

Bossuyt, F. & M. C. Milinkovitch (2000): Convergent adaptive radiations in Madagascan and Asian ranid frogs reveal covariation between larval and adult traits. – Proceedings of the National Academy of Sciences of the U.S.A., **97**: 6585–6590.

Bruford, M. W., O. Hanotte, J. F. Y. Brookfield & T. Burke (1992): Single-locus and multilocus DNA fingerprint. – pp. 225–270 in Hoelzel, A. R. (ed.): Molecular Genetic Analysis of Populations: A Practical Approach. IRL Press, Oxford.

Dalbeck, L., M. Hachtel & A. Heyd (1997): Amphibien im Rhein-Sieg-Kreis und in der Stadt Bonn. Verbreitung, Gewässerpräferenzen, Vergesellschaftung und Gefährdung. – Decheniana, **150**: 235–292.

Dufresnes, C., L. Bonato, N. Novarini, C. Betto-Colliard, N. Perrin & M. Stöck (2014): Inferring the degree of incipient speciation in secondary contact zones of closely related lineages of Palearctic green toads (*Bufo viridis* subgroup). – Heredity, **113**: 9–20.

Earl, D. A. & B. M. von Holdt (2012): STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. – Conservation Genetic Resources, **4**: 359–361.

Evanno, G., S. Regnaut & J. Goudet (2005): Detecting the number of clusters of individuals using the software STRUCTURE. A simulation study. – Molecular Ecology, **14**: 2611–2620.

Excoffier, L., G. Laval & S. Schneider (2005): Arlequin (version 3.0). An integrated software package for population genetics data analysis. – Evolutionary Bioinformatics Online, **1**: 47.

Fouquet, A., E. A. Courtois, D. Baudain, J. Dias Lima, S. Marques Souza, B. P. Noonan & M. T. Rodrigues (2015): The trans-riverine genetic structure of 28 Amazonian frog species is dependent on life history. – Journal of Tropical Ecology, **31**: 361–373.

Gascon, C., J. R. Malcolm, J. L. Patton, M. N. F. da Silva, J. P. Bogart, S. C. Lougheed, C. A. Peres, S. Neckel & P. T. Boag (2000): Riverine barriers and the geographic distributions of Amazonian species. – Proceedings of the National Academy of Sciences of the United States of America, **97**: 13672–13677.

Gehring, P. S., M. Pabijan, J. E. Randrianirina, F. Glaw & M. Vences (2012): The influence of riverine barriers on phylogeographic patterns of Malagasy reed frogs (*Heterixalus*). – Molecular Phylogenetics and Evolution, **64**: 618–632.

Geil, W. (1962): Blüte und Ende einer Population von *Bufo viridis*. – Deutsche Aquarien- und Terrarienzeitschrift, **15**: 254–255.

Glaw, F. & M. Vences (1989): Zur Verbreitung von Wechselkröte (*Bufo viridis* Laurenti, 1768) und Kreuzkröte (*Bufo calamita* Laurenti, 1768) im Nördlichen Rheinland. – Jahrbuch für Feldherpetologie **3**: 61–75.

Glaw, F. & M. Vences (1991): Wechselkröte – *Bufo v. viridis* Laurenti 1768. – pp. 53–56 in Mittman, R. & K. Simon (eds): Die Amphibien und Reptilien im Raume Köln. – Arbeitsgruppe „Amphibien und Reptilienschutz Köln", 109 pp.

Günther, R. & R. Podloucky (1996): Wechselkröte – *Bufo viridis* Laurenti, 1768. – pp. 322–343 in: Günther, R. (ed.): Die Amphibien und Reptilien Deutschlands. – Jena, Stuttgart, Lübeck, Ulm (Gustav Fischer), 825 pp.

Moritz, C. (1994): Defining 'Evolutionarily Significant Units' for conservation. – Trends in Ecology and Evolution, **9**: 373–375.

Palumbi, S., A. Martin, S. Romano, W. O. McMillan, L. Stice & G. Grabowski (1991): The simple fool's guide to PCR. – Department of Zoology, Honolulu.

Perl, R. G. B., E. Geffen, Y. Malka, A. Barocas, S. Renan, M. Vences & S. Gafny (2018): Population genetic analysis of the recently rediscovered Hula painted frog (*Latonia nigriventer*) reveals high genetic diversity and low inbreeding. – Scientific Reports**, 8**: 5588.

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

Rice, W. R. (1989): Analysing tables of statistical tests. –Evolution, **43**: 223–225.

Schmidt, E. & K. Simon (2017): Artenschutz für die Wechselkröte in Köln. – Feldherpetologisches Magazin, **8**: 27–35.

Schuelke, M. (2000): An economic method for the fluorescent labeling of PCR fragments. – Nature Biotechnology, **18**: 233–234.

Selkoe, K. A. & R. J. Toonen (2006): Microsatellites for ecologists. A practical guide to using and evaluating microsatellite markers. – Ecology Letters, **9**: 615–629.

Speybroeck, J., W. Beukema, B. Bok, J. dervan Voort & I. Velikov (2016): Field guide to the amphibians and reptiles of Britain and Europe. – London & New York: Bloomsbury.

Stöck, M., G. Grifoni, N. Armor, U. Scheidt, A. Sicilia & N. Novarini (2016): On the origin of the recent herpetofauna of Sicily: Comparative phylogeography using homologous mitochondrial and nuclear genes. – Zoologischer Anzeiger, **261**: 70–81.

Stöck, M., D. K. Lamatsch, C. Steinlein, J. T. Epplen, W.-R. Grosse, R. Hock, T. Klapperstück, K. P. Lampert, U.Scheer, M. Schmid, M. Schartl (2002): A bisexually reproducing all-triploid vertebrate. – Nature Genetics, **30**: 325–328.

Stöck, M., C. Moritz, M. Hickerson, D. Frynta, T. Dujsebayeva, V. Eremchenko, J. R. Macey, T. J. Papenfuss & D. B. Wake (2006): Evolution of mitochondrial relationships and biogeography of Palearctic green toads (*Bufo viridis* subgroup) with insights in their genomic plasticity. – Molecular Phylogenetics and Evolution, **41**: 663–689.

Stöck, M., P. Roth, R. Podloucky & K. Grossenbacher (2009): Wechselkröten. – pp. 413–498 in: Grossenbacher, K. (ed.): Handbuch der Reptilien und Amphibien Europas, Band 5/II. – Wiebelsheim (Aula).

Stöck, M., A. Sicilia, N. M. Belfiore, D. Buckley, S. Lo Brutto, M. Lo Valvo & M. Arculeo (2008): Post-Messinian evolutionary relationships across the Sicilian channel: Mitochondrial and nuclear markers link a new green toad from Sicily to African relatives. – BMC Evolutionary Biology, **8**: 56.

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

Vences, M., J. Becker, H. Sauer & F. Glaw (2003a): Verbreitung und Bestandssituation der Wechselkröte (*Bufo viridis*) in Nordrhein-Westfalen. – pp. 77–84 in: Podloucky, R. & U. Manzke (eds): Verbreitung und Ökologie der Wechselkröte (*Bufo viridis*). – Mertensiella, **14**.

Vences, M., F. Glaw & M. Franzen (2003b): Perspektiven für den kostengünstigen Erhalt von Lebensräumen in Abgrabungen und ihre Bedeutung für die Wechselkröte (*Bufo viridis*). – Mertensiella, **14**: 316–32.

Vences, M., F. Glaw & M. Hachtel (2011): Wechselkröte – *Bufo viridis*. – pp. 667–688 in: Hachtel, M., M. Schlüpmann, K. Weddeling, B. Thiesmeier, A. Geiger & C. Willigalla for the Arbeitskreis Amphibien und Reptilien NRW (eds): Handbuch der Amphibien und Reptilien Nordrhein-Westfalens. – Laurenti Verlag.

Wollenberg Valero, K.C. (2015): Evidence for an intrinsic factor promoting landscape genetic divergence in Madagascan leaf-litter frogs. – Frontiers in Genetics, **6**: 155.

Supplementary material

Supplementary Figure S1. Comparison of STRUCTURE runs from K=1 to K=8.

Supplementary Figure S2. Barplot from an analysis with STRUCTURE excluding locus Bvir16861 with K=5 for 84 individuals.

Supplementary Table S1. Full microsatellite library.

Supplementary Table S2. List of *Bufotes viridis* specimens sampled for population genetic analysis.

Supplementary Table S3. Number of specimens per site sequenced for fragments of the mitochondrial 16S and cytochrome b genes.

Supplementary Table S4. Expected (He) and observed (Ho) heterozygosities for all microsatellite loci and sites sampled for *Bufotes viridis*.