SALAMANDRA **53**(2) | 201–211 | 15 May 2017 | ISSN 0036–3375

Conservation genetics of *Bombina v. variegata* (Anura: Bombinatoridae) in northern Hesse, Germany

Daniela Guicking[1], Lennart Finke[1], Martin Wittich[1], Ina Pfeiffer[1], Michael Veith[2], Christian Geske[3], Ulrich Braukmann[4], Kurt Weising[1] & Claus Neubeck[4]

[1)] University of Kassel, FB 10, Molecular Systematics of Plants, Heinrich-Plett-Str. 40, 34132 Kassel, Germany

[2)] University of Trier, Department of Biogeography, Universitätsring 15, 54296 Trier, Germany

[3)] Hessisches Landesamt für Naturschutz, Umwelt und Geologie, Abteilung Naturschutz, Europastr. 10, 35394 Gießen, Germany

[4)] University of Kassel, FB 06, Aquatic Ecology and Water Resources Development, Nordbahnhofstr. 1a, 37213 Witzenhausen, Germany

Corresponding author: Daniela Guicking, e-mail: guicking@uni-kassel.de

Manuscript received: 13 November 2015

Accepted: 18 December 2015 by Alexander Kupfer

Abstract. We report the genetic diversity and population structure of the Yellow-bellied Toad (*Bombina variegata*) at the northern edge of its distribution range in northern Hesse, Germany. A total of 281 samples from 20 populations were analysed, using six polymorphic nuclear microsatellite markers (ncSSRs). Moderate genetic diversity (H_e = 0.37–0.59) was detected within these *B. variegata* populations. We found evidence of a distinctive population structure, with populations at the edge of the study area showing lower degrees of diversity and higher degrees of isolation than more central populations. No genetic differentiation was found between populations from the middle Fulda and Werra river valleys, suggesting that fragmentation, e.g., by the A4 highway that separates the two river valleys, has not yet had genetic consequences. Furthermore, indications of an isolation-by-distance pattern was found, suggestive of restricted gene flow between the studied populations. To ensure the long-term survival of the Yellow-bellied Toad in northern Hesse, we recommend continuous management efforts that focus on the reconnection of isolated populations and continuation of demographic population monitoring supplemented by population genetic analyses.

Key words. Amphibia, *Bombina variegata variegata*, genetic diversity, nuclear microsatellite markers, population structure.

Introduction

The Yellow-bellied Toad, *Bombina variegata* (Linnaeus, 1758), is a small European anuran adapted to living within networks of small, insolated, ephemeral water bodies for reproduction (Gollmann & Gollmann 2012). Such habitat networks are naturally found in floodplains of streams and rivers, but the species' predilection for mountainous and forested landscapes of central and southeastern Europe may also indicate an adaptation to networks of small and ephemeral water bodies in forest habitats, such as footprints and wallows of large herbivores such as wild boar, red deer and extinct mega-herbivores (e.g., Veith 1996, Schlüpmann et al. 2011, Gollmann & Gollmann 2012). It is sister species to the lowland-adapted fire-bellied toad *Bombina bombina* (Linnaeus, 1761) (e.g., Fromhage et al. 2004, Hofman et al. 2007, Pabijan et al. 2013), which is distributed farther to the north and east.

In southern Lower Saxony, Germany, the Yellow-bellied Toad reaches its northernmost distribution limits (Bundesamt für Naturschutz 2014). *Bombina variegata* is categorized as "Least Concern" in the IUCN Red List of Threatened Species (IUCN 2015). In Germany, it has suffered severe population declines during the last decades and is considered Seriously Endangered ("stark gefährdet"; Kühnel et al. 2009).

The most serious threats to the Yellow-bellied Toad are seen in conjunction with anthropogenic alterations of their aquatic habitats (Gollmann & Gollmann 2012). Habitat loss, which in the past especially affected small and ephemeral water bodies, is considered the main reason for the decline of *B. variegata* in Central Europe. Another problem for population survival is that dynamic processes of natural river systems have to a large extent been suppressed by anthropogenically reshaped riverbanks and the loss of floodplains. Fragmentation of habitats through urbanisation, roads, railway lines, and agricultural land use have led to the fragmentation and decline of formerly connected populations and today inhibit genetic exchange between populations. Consequently, most remaining populations are confined to anthropogenic habitats such as quarries, gravel pits, and military training grounds, and their sur-

vival strongly depends on supportive measures (Bundesamt für Naturschutz 2014).

To improve the situation of the Yellow-bellied Toad in Germany, conservation projects have been initiated, e.g., in Hesse, North Rhine-Westphalia, Bavaria, and Baden-Württemberg (Bundesamt für Naturschutz 2014). In northern Hesse, population monitoring and conservation measures began more than 30 years ago. Conservation efforts concentrate on habitat restoration, including establishing artificial ponds for spawning, clearing of overgrown areas, and creating hibernation sites. Nicolay & Nicolay (2013) provide an up-to-date review of the status of the Yellow-bellied Toad in one district within the project's range in northern Hesse and comment on conservation strategies. For the period of 2001–2007, the conservation status of the populations of the Yellow-bellied Toad in Hesse was poor (Bütehorn et al. 2010). Therefore, the provincial government initiated a species action plan in 2007 to improve this situation, especially by managing the most important habitats (Malten & Steiner 2008, Geske 2009). For the long-term survival of stable populations, revitalizing of floodplains, less extensive grazing, and reconnecting habitat fragments may be most promising measures (Bundesamt für Naturschutz 2014).

In 2011, a conservation project titled "Die Gelbbauchunke als Leitart für Pionieramphibien in den Flussauen Nordhessens – Naturschutzgenetik, Populationsökologie und Schutzmaßnahmen" ["The Yellow-bellied Toad as an indicator species for the revitalisation of submontane floodplains in northern Hesse – conservation genetics, population ecology, and conservation measures"] was established to complement existing efforts for the conservation of the Yellow-bellied Toad in Hesse (Neubeck & Brauckmann 2014). It was in the framework of this project that we conducted a population genetic survey of *B. variegata* based on nuclear microsatellite markers in order to evaluate the genetic situation of *B. variegata* within the study area and provide a basis for the conservation and management of this endangered species in northern Hesse. These data complement the only two other studies so far published on conservation genetics of the Yellow-bellied Toad in Lower Saxony, Germany (Weihmann et al. 2009) and northern Italy (Cornetti 2013), respectively.

More specifically, the objectives of our study were to (1) describe the genetic diversity of populations of the Yellow-bellied Toad in northern Hesse, (2) identify populations that show indications of inbreeding or a recent bottleneck, (3) examine how its genetic diversity is spatially structured, and (4) delineate manageable units in the Yellow-bellied Toad in northern Hesse.

Material and methods

Sampling

A total of 307 individuals of *B. variegata* were sampled at 20 localities in northern Hesse, Germany, between May and October of 2011 and during one day in June of 2012 (Fig. 1). Only animals larger than 2 cm were collected as this minimized the risk of sampling young-of-the-year offspring at a single pond that potentially represented full siblings whose inclusion would compromise the population genetic results. For tissue sampling, we followed the minimally invasive method described by Poschadel & Möller (2004). The animals were captured in the field, held between two fingers, and the mouth was cautiously opened using a flat wooden spatula. An ordinary cotton pad was used to swab the oral cavity. Two samples were taken from each animal. The swabs were immediately stored at -20°C in 1.5 ml Eppendorf tubes.

The ventral patterns of all animals were photographed for individual identification and capture-recapture-analyses (N. Wagner et al. unpubl. data). All animals were weighed and their sexes identified by checking for nuptial pads characteristic of males.

For data analysis, animals from very proximal localities were pooled into a single population if their migration between localities was proven by capture-recapture data. This was the case for the three localities Baumbach Herrenwiese, Steinbruch Hergershausen, and Mergelgrube Baumbach, which were combined in a single population Mergelgrube (MER+), and Alte Fulda Blankenheim, Alte Fulda Blankenheim Randsenke, and Nasse Wiesen bei Meckbach, which were combined in the population Blankenheim (BLA+) (Fig. 1).

Laboratory analysis

Total DNA was isolated from the swabs using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. Extracted DNA was stored at -20°C until further processing.

For population genetic analysis, ten autosomal microsatellite loci were analysed using primers as published by Stuckas & Tiedemann (2006) and Hauswaldt et al. (2007) for *Bombina bombina*. We selected the same ten markers (9H, F22, 12F, B14, B13, 5F, 1A, 10F, F2, 8A) as Weihmann et al. (2009). Except for F2, these markers, and two more from Nürnberger et al. (2003), were also used by Cornetti (2013).

PCR amplification was performed in a 10 µl reaction volume containing 10–100 ng of total genomic DNA, 0.4 µM each of forward and reverse primer, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 0.02 u/µl of DNA polymerase (Phusion High Fidelity DNA polymerase F-530, Thermo Scientific), and 1 × reaction buffer (F-518 Phusion HF reaction buffer, Thermo Scientific). After an initial denaturation step (94°C, 3 min), 35 cycles were performed at 94°C for 30 s, the locus-specific annealing temperature for 30 s, and 72°C for 30 s. A final elongation step at 72°C for 10 min followed. Optimal annealing temperatures as determined from a gradient PCR were 56.2°C for 9H, F22 and 12F, 62.6°C for B14, 53.0 for B13, 58.9 for 5F, 60.9 for 1A, 60.3 for F2, and 63.5 for 10F and 8A. The amplified products were genotyped on an ABI Prism 310 Genetic Analyser (Applied Biosystems). Differential labelling of the primers al-

lowed analysing three loci plus a size standard (350 TAMRA Size Standard; Applied Biosystems, GeneScan) in each run. Data were collected with 310 Data Collection Software v. 3.1.0, and allele lengths were measured with the help of GeneScan Analyzer 3.7 and ABI Prism Genotyper 2.5.

All loci were tested for two-locus linkage disequilibrium (LD). Indications of a possible LD were found for five locus pairs in two or three out of 14 populations tested. Because the few significant results were not consistent over populations or loci, we assumed that genotypes at one locus were independent of those at the other loci (see also Weihmann et al. 2009, Cornetti 2013). All loci were further tested for the presence of null alleles, allelic drop-out and scoring errors using MicroChecker 2.2.3 (van Oosterhout et al. 2004).

Data analysis

Population genetic analysis of microsatellite data was performed using various software packages. To examine within-population genetic diversity, the mean number of alleles per locus and allelic richness (a measure of the number of alleles standardized for sample size, in our study, five; El Mousadik & Petit 1996) were calculated with the software Fstat 2.9.3.2 (Goudet 2002). Mean observed and expected heterozygosities (H_o and H_e; Nei 1987) and the inbreeding coefficient (F_{IS}) were obtained with Arlequin 3.5.1.2 (Excoffier & Lischer 2010). Private alleles were detected with the software Convert 1.31 (Glaubitz 2004).

To detect recent genetic bottlenecks, the software Bottleneck 1.2.02 (Cornuet & Luikart 1997) was used with default settings. We considered the results of a Wilcoxon's sign rank test, which is based on heterozygosity excess, using the stepwise mutation model (SMM) and the two-phase model (TPM), as recommended by Luikart & Cornuet (1998), and results of the mode-shift test that evaluates the allele frequency distribution (Cornuet & Luikart 1997).

To assess the effective population size, N_e, we used an approximate Bayesian computation analysis as incorporated in ONeSAMP 1.2 (Tallmon et al. 2004, Tallmon et al.

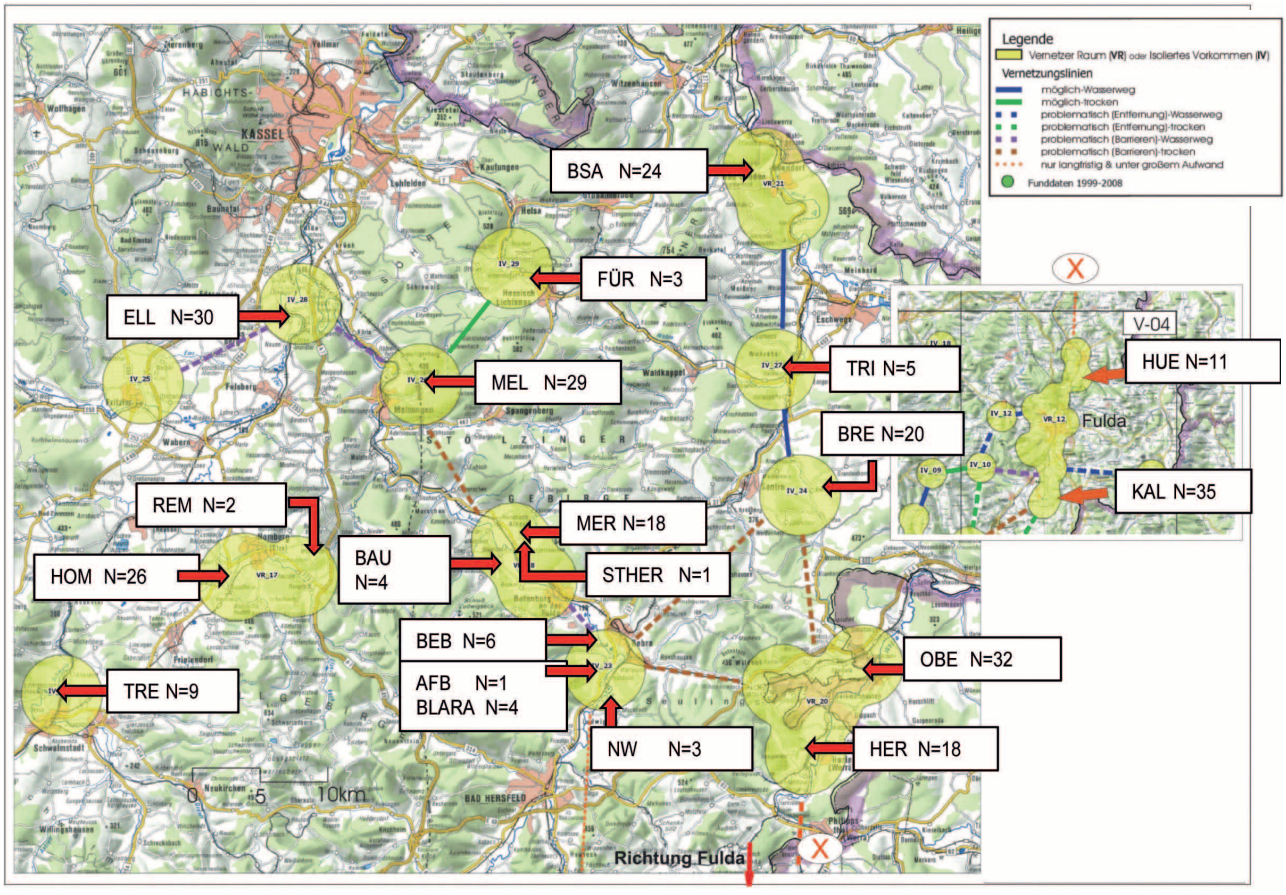

Figure 1. Geographic locations of twenty sample populations of *B. variegata* in northern Hesse. Abbreviations: Werra-Meißner district: Für – Fürstenhagen; BSA – Bad Sooden-Allendorf; BRE – Breitau; TRI – Trimberg. Hersfeld Rotenburg district, central Werra region: OBE – Obersuhler Aue; HER – Heringen, Obere Aue. Hersfeld Rotenburg district, central Fulda valley: MER – Mergelgrube Baumbach; STHER – Steinbruch Hergershausen; BAU – Baumbach Herrenwiese; BEB – Bebra Kiesgrube; AFB – Alte Fulda Blankenheim; BLARA – Alte Fulda Blankenheim Randsenke; NW – Nasse Wiesen bei Meckbach. Schwalm-Eder district: ELL – Ellenberg; MEL – Melsungen; HOM – Homberg/Efze; REM – Remsfeld; TRE – Treysa, Hardtberg. Fulda district: HUE – Hünfeld-Rückers; KAL – Kalbach Deponie Schrimpf.

2008). ONeSAMP cannot process multiple missing-data instances per sample, for which reason all individuals with data missing at more than one locus were excluded. The presumed lower and upper limits of N_e were set to 2 and 100, respectively.

To identify the amount of genetic variation attributable to within- and between-population variation, an analysis of molecular variance (AMOVA; Excoffier et al. 1992) was calculated for unordered alleles with Arlequin 3.5.1.2, including populations with N > 10. The same software was used to estimate pairwise F_{ST}-values and test their significance using 10,000 permutations. Because F_{ST} of Wright (1943) strongly depends on the observed within-population diversity (Charlesworth 1998, Hedrick 1999), we also calculated an unbiased standardized estimator G'_{ST} (Hedrick 2005) using GenAlEx 6.5 (Peakall & Smouse 2012). Whitlock (2011), however, showed that both estimates of pairwise genetic differentiation may be misleading when calculated from markers with high mutation rates such as microsatellites. While F_{ST} usually underestimates the differentiation when the mutation rate is high, G'_{ST} will overestimate it (Whitlock 2011).

To examine whether pairwise genetic distances and geographic distances are correlated between populations with N > 10 (isolation-by-distance), the significance of the rank correlation coefficient was tested using the software Isolde incorporated in Genepop (Raymond & Rousset 1995).

We used Structure 2.3.4 (Pritchard et al. 2000) to estimate the number of distinct genetic clusters (K) by grouping individuals into 1–10 groups. The admixture model without prior information on sample population information was applied, and allele frequencies were allowed to be correlated among clusters (Falush et al. 2003). Ten independent runs were performed for each K (500,000 iterations per run with a burn-in of 50,000 iterations). The most likely number of genetic clusters (K) was chosen by calculating the ΔK statistic of Evanno et al. (2005) with the help of Structure Harvester (Earl & vonHoldt 2012).

Where called for, a sequential Bonferroni correction was applied to correct for multiple statistical tests (Rice 1988).

Results

Microsatellite analysis

Four of the ten loci used by Weihmann et al. (2009) turned out monomorphic in our study (loci B13, 1A, F22, and F2) and thus were excluded from the analysis. The other six loci (9H, B14, 12F, 5F, 10F, 8A) yielded 4 to 10 alleles per locus (average 6.3 alleles per polymorphic locus, Table 1). Analyses with MicroChecker yielded no indications of allelic drop-outs. Possible null alleles were detected at locus 9H in Bad Sooden-Allendorf and Treysa, locus 8A in Kalbach, and locus B14 in Bad Sooden-Allendorf, Obersuhler Aue, Heringen, Ellenberg, and Homberg/Efze. Scoring errors due to stuttering might have resulted in an excess of homozygotes at locus B14, the only locus with a dinucleotide repeat motif (the other microsatellites consisted of tetranucleotide repeat motifs). Due to the already low number of polymorphic loci we decided to include all six polymorphic loci in the analysis in spite of the mentioned inconsistencies.

Table 1. Genetic diversity of ten ncSSR loci used for analysis in *B. variegata*. Indicated are the locus names, number of alleles (A), size range of alleles, and observed heterozygosities (H_o) in our study, the study of Cornetti (2013) and the study of Weihmann et al. (2009). Note that interpretation of the absolute fragment lengths differed by one base pair at some loci between studies, and that Cornetti (2013) did not provide values for H_o.

	B. variegata in northern Hesse (this study, N=281)			*B. variegata* in Italy (Cornetti 2013, N=200)		*B. variegata* in Lower Saxony (Weihmann et al. 2009, N=150)		
Locus	A	Size range	H_o	A	Size range	A	Size range	H_o
F22	1	143	n.a.	2	142–148	6	137–169	0.04
B14	6	160–172	0.40	5	164–172	6	138–200	0.48
B13	1	115	n.a.	3	114–134	13	95–161	0.24
5F	9	115–163	0.54	3	116–148	10	91–163	0.52
9H	4	151–163	0.32	6	156–176	9	119–203	0.58
F2	1	468	n.a.			10	270–378	0.17
1A	1	323	n.a.	2	322–326	8	323–383	0.09
8A	10	283–339	0.53	6	291–331	11	291–363	0.48
10F	5	209–225	0.58	7	206–230	7	193–229	0.71
12F	4	143–163	0.37	8	219–247	6	213–233	0.50

No PCR products could be obtained for three or more loci from nine of the 307 animals sampled. Another 17 specimens turned out to be recaptures, as could be validated by comparing photographs of their individual-specific ventral colour patterns. Therefore, data from a total of 281 animals were included in the final analyses.

A comparison of the allele lengths inferred during the two typing approaches of 17 recaptured animals allowed to estimate the genotyping error rate. Of the six loci typed twice in 17 individuals, allele lengths were differently interpreted in five cases, suggesting a genotyping error rate of about 5%. Interestingly, none of these errors occurred at the dinucleotide repeat locus B14, for which MicroChecker suggested possible typing errors.

Genetic variation within populations

All populations showed moderate levels of within-population genetic diversity. Total allele numbers over six loci ranged from 13 to 25, and levels of expected heterozygosity ranged from 0.37 to 0.59 (Table 2). The population with the lowest diversity was the one from Homberg/Efze in the west of our study area, while highest levels of population diversity were found in Obersuhler Aue, Heringen, and Mergelgrube, all living in the centre of our study area.

Kalbach, in the south of our study area, was the only population with a significantly lower observed than expected heterozygosity and thus significantly elevated in-

Table 2. Genetic diversity of 16 *Bombina variegata* populations in northern Hesse: sample size (N), number of alleles (A), allelic richness (AR; calculated for a minimum of five individuals per population), observed (H_o) and expected heterozygosity (H_e), inbreeding coefficient (F_{IS}), number of private alleles (PA), frequencies of private alleles (Freq. PA), bottleneck test statistics: one-tailed Wilcoxon's test SMM (BN $W1t_{SMM}$), one-tailed Wilcoxon's test TPM (BN $W1t_{TPM}$), mode shift test (BN MS), estimates of effective population size, N_e (ONeSAMP) with 95% credible limits for the posterior distribution. Diversity estimates are shown as averages ± standard deviation. For bottleneck analysis, only significant statistics without Bonferroni correction are shown. ** significance level $p < 0.01$. – not calculated due to very small N.

Population	N	A	AR	H_o	H_e	F_{IS}	PA	Freq. PA	BN $W1t_{SMM}$	BN $W1t_{TPM}$	BN MS	N_e (ONeSAMP)
FÜR	3	–	–	–	–	–	–	–	–	–	–	–
BSA	24	19	2.58±0.62	0.41±0.18	0.50±0.15	0.11	1	0.21				13.8 (10.3–20.6)
BRE	20	16	2.31±0.63	0.42±0.18	0.44±0.15	0.05	0	0				16.2 (11.7–27.5)
TRI	5	15	2.50±0.55	0.70±0.32	0.51±0.19	-0.47	0	0	–	–	–	–
OBE	32	25	3.01±0.95	0.51±0.14	0.58±0.17	0.01	0	0		0.016		28.4 (22.0–47.2)
HER	18	25	3.13±0.83	0.43±0.23	0.56±0.20	0.10	2	0.09				17.0 (13.1–26.5)
MER+	23	25	3.06±0.82	0.61±0.11	0.59±0.12	-0.07	1	0.02				17.8 (14.3–27.0)
BEB	6	16	2.58±0.94	0.53±0.25	0.50±0.26	-0.05	1	0.08	–	–	–	–
BLA+	8	15	2.42±0.61	0.46±0.26	0.52±0.22	0.02	0	0	–	–	–	–
ELL	30	20	2.36±0.61	0.44±0.31	0.41±0.20	-0.21	1	0.03				11.6 (8.9–16.3)
MEL	29	17	2.26±0.59	0.42±0.24	0.40±0.20	-0.06	1	0.03				
HOM	26	13	2.01±0.78	0.38±0.26	0.37±0.23	-0.05	0	0	0.031	0.031	shifted	20.8 (14.3–35.2)
REM	2	–	–	–	–	–	–	–	–	–	–	–
TRE	9	19	2.60±0.49	0.46±0.32	0.46±0.17	0.03	0	0	–	–	–	–
HUE	11	15	2.32±0.81	0.52±0.24	0.45±0.22	-0.18	0	0			shifted	11.5 (8.8–16.6)
KAL	35	18	2.33±0.84	0.33±0.15	0.40±0.20	0.19 **	1	0.01				16.6 (12.2–24.6)

breeding coefficient ($F_{IS} = 0.19$, $p > 0.01$, Table 2). Few private alleles were found, most of which occurred at low frequencies. Only one private allele, found in the Bad Sooden-Allendorf population, showed a high frequency (21%), suggesting reduced gene flow (Table 2).

Estimates of recent effective population size N_e were low, ranging from 11.5 (Hünfeld-Rückers, Ellenberg) to 28.4 (Obersuhler Aue) (Table 2). The upper value of the 95% credible limit for the posterior distribution did not exceed 50 in any of the populations examined. Consistent results for the occurrence of a recent bottleneck detected over all three tests employed were obtained only for the population in Homberg/Efze (Table 2). Distortion from an L-shaped allele frequency distribution in Hünfeld and another significant result revealed by the two-phase mutation model (TPM) of Wilcoxon's test in Obersuhler Aue were not supported by the remaining tests and thus were regarded unreliable.

Population structure

An AMOVA was performed to estimate the relative contribution of within- and among-population variation. The AMOVA assigned 79.4% of the total variance to within-population variation, and 20.6% to among-population variation equivalent to a highly significant global F_{ST}-value of 0.206 ($p < 0.001$), which indicated a high overall genetic differentiation among populations. Pairwise F_{ST}-values among populations ranged from 0.00 to 0.42 (Table 3) and showed a positive correlation with geographic distances ($p = 0.043$, Fig. 2), indicating a weak isolation-by-distance pattern. Most pairwise F_{ST}-values were relatively high and significant, with the few exceptions referring to comparisons between populations from the Middle Fulda and Werra river valleys (Bebra, Blankenheim, Obersuhler Aue, Heringen). In the cases of these four populations, no clear genetic differentiations were found, indicating genetic connectivity, although we have to point out that this result is based on small population sizes in Bebra and Blankenheim.

To estimate the number of genetic units within *B. variegata* in northern Hesse, a Bayesian cluster analysis was per-

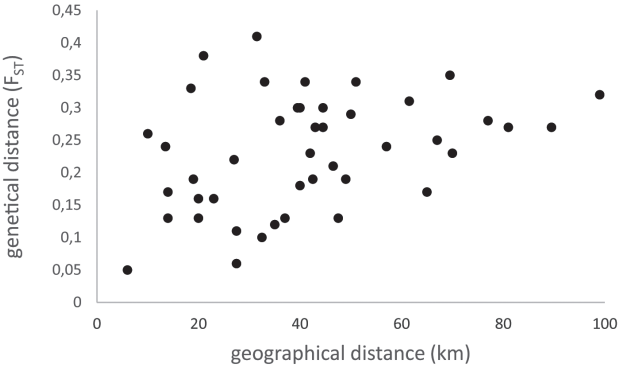

Figure 2. Relationship between geographical and genetic distances between populations with N > 10 of *B. variegata* in northern Hesse.

Table 3. Geographical distances (km) (above diagonal) and pairwise genetic divergence between populations (F_{ST} values above, G'_{ST} values below) (below diagonal). Significant F_{ST} values are shown in bold, non-significant values are shown in italics.

	BSA	BRE	TRI	OBE	HER	MER+	BEB	BLA+	ELL	MEL	HOM	TRE	HUE	KAL
BSA		14.0	23.5	37.0	42.5	32.5	36.0	42.0	36.0	33.0	47.5	63.0	67.0	99.0
BRE	**0.17** 0.25		9.5	14.0	19.0	20.0	18.0	23.0	40.0	31.5	42.0	55.0	44.5	77.0
TRI	**0.12** 0.19	*0.08* 0.12		22.5	28.5	23.0	23.5	28.5	35.5	27.5	43.0	57.0	55.0	87.0
OBE	**0.12** 0.22	**0.13** 0.21	**0.17** 0.32		6.0	27.5	17.0	19.0	49.0	39.5	46.5	58.0	35.0	65.0
HER	**0.19** 0.33	**0.19** 0.30	**0.23** 0.42	**0.05** 0.09		27.5	16.0	16.5	50.0	41.0	43.0	53.5	27.0	57.0
MER+	**0.10** 0.18	**0.13** 0.21	**0.08** 0.16	**0.06** 0.12	**0.11** 0.21		12.0	14.0	23.0	13.5	20.0	34.0	40.0	70.0
BEB	**0.13** 0.20	*0.09* 0.12	**0.19** 0.34	*0.00* 0.00	*0.04* 0.06	**0.08** 0.15		4.0	35.5	25.0	28.0	42.0	32.0	62.0
BLA+	**0.13** 0.26	**0.19** 0.31	**0.24** 0.43	*0.00* 0.02	*0.07* 0.14	**0.06** 0.17	*0.04* 0.07		37.0	27.0	28.5	42.0	29.0	59.0
ELL	**0.28** 0.44	**0.30** 0.44	**0.21** 0.30	**0.19** 0.31	**0.29** 0.47	**0.16** 0.26	**0.30** 0.45	**0.19** 0.30		10.0	21.0	33.0	61.5	89.5
MEL	**0.33** 0.54	**0.41** 0.62	**0.31** 0.46	**0.30** 0.52	**0.34** 0.54	**0.24** 0.40	**0.42** 0.66	**0.34** 0.56	**0.26** 0.36		18.5	33.0	51.0	81.0
HOM	**0.13** 0.18	**0.23** 0.30	**0.24** 0.31	**0.21** 0.32	**0.27** 0.39	**0.16** 0.23	**0.24** 0.31	**0.25** 0.39	**0.38** 0.53	**0.33** 0.44		16.0	44.5	69.5
TRE	**0.15** 0.22	**0.25** 0.36	**0.14** 0.23	**0.22** 0.39	**0.28** 0.49	**0.10** 0.16	**0.26** 0.44	**0.27** 0.45	**0.32** 0.48	**0.25** 0.34	**0.21** 0.26		48.0	67.0
HUE	**0.25** 0.41	**0.27** 0.40	**0.28** 0.48	**0.12** 0.20	**0.22** 0.38	**0.18** 0.32	**0.17** 0.26	**0.16** 0.29	**0.31** 0.45	**0.34** 0.49	**0.30** 0.40	**0.26** 0.41		20.0
KAL	**0.32** 0.50	**0.28** 0.41	**0.29** 0.42	**0.17** 0.29	**0.24** 0.38	**0.23** 0.39	**0.28** 0.40	**0.25** 0.42	**0.27** 0.38	**0.27** 0.36	**0.35** 0.49	**0.36** 0.53	**0.26** 0.37	

formed using the software Structure. The highest likelihoods and ΔK statistics were obtained for K = 2 and K = 6 clusters (Fig. 3). As for K = 2, most populations exhibited a clearly admixed origin, with only the individuals from Bad Sooden-Allendorf and Melsungen being referrable to one of the two clusters (Fig. 3). As for K = 6, the relative proportions of each cluster varied widely among populations. Populations in Melsungen, Ellenberg, and Kalbach at the edge of our study area appeared clearly isolated in that their individuals were predominantly assignable to a single cluster that was not strongly represented in other populations. The remaining populations, especially in the central part of our study area (Mergelgrube, Bebra, Blankenheim, Obersuhler Aue, Heringen Hünfeld-Rückers), comprised a mix of genetic clusters and similar patterns of cluster affiliation, indicating at least some degree of genetic connectivity.

Discussion

Genetic diversity of populations, inbreeding, and bottlenecks

Genetic diversity of a population is dependent on population size, including historical demographics, the extent of gene flow between populations, the reproductive system, and natural selection (Lowe et al. 2004). The Yellow-bellied Toad in central Germany descended from animals that survived the last Pleistocene cold period in refugia on the Balkans (Hofmann et al. 2007, Fijarczyk et al. 2011). Successive bottlenecks during postglacial northward range expansion, as well as small population sizes, and reduced gene flow through isolation at the northern edge of the distribution range, are likely to have resulted in reduced genetic variation (Taberlet et al. 1998, Hewitt 2004).

Genetic investigations of *B. variegata* in northern Hesse revealed within-population diversities with expected heterozygosities in the range of 0.37–0.59 (Table 2). Weihmann et al. (2009) reported a slightly lower range of expected heterozygosities (0.31–0.53) in the isolated populations in Lower Saxony, and Cornetti (2013) reported values of expected heterozygosities in the range of 0.34–0.54 for northern Italian *B. variegata*. Also, ranges of effective population sizes, N_e, were similarly low in northern Hesse and Italy (Cornetti 2013). Most estimates of N_e were smaller than 20, suggesting very low effective population sizes as compared to many other anuran species (e.g., Schmeller & Merilä 2007, Phillipsen et al. 2011).

A notably reduced genetic diversity was found in Homberg/Efze, the only population that also exhibited indications of a recent population bottleneck (Table 2). It is locat-

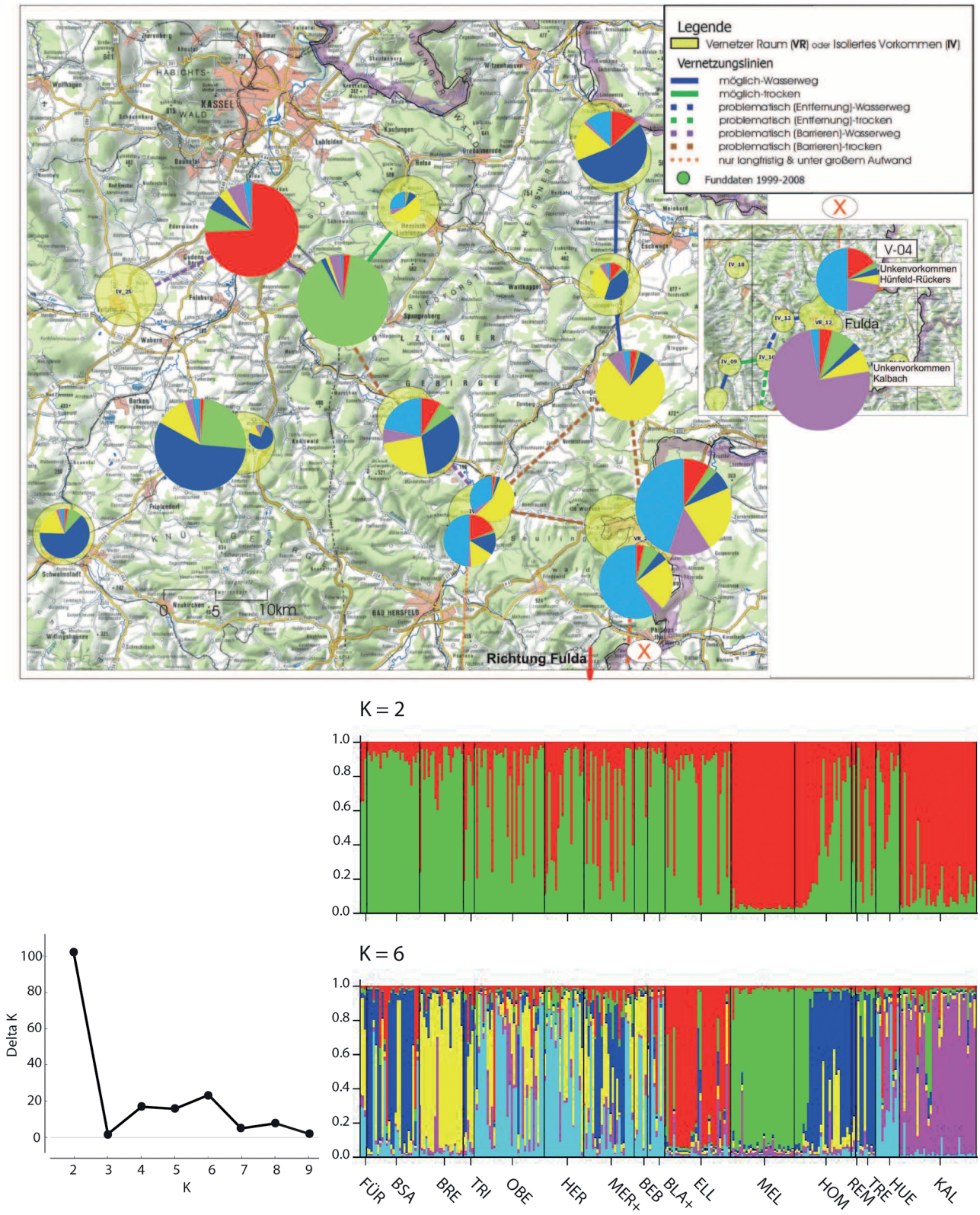

Figure 3. Results of a Bayesian cluster analysis of *B. variegata* in northern Hesse. Above: Pie charts show the geographic distribution of relative proportion of ancestry in each of K = 6 genetic clusters for the 16 study populations. Below left: Delta K plot as obtained from Structure Harvester. Below right: Bar plots of proportion of ancestry of each sampled individual for two (K = 2) and six (K = 6) genetic clusters.

ed in former military training grounds. It has always been rather small and greatly suffered when the area was abandoned by the German armed forces in the early 1990s (D. Schmidt pers. comm.). Possibly due to the lack of ephemeral water bodies for reproduction, which had previously formed in the tracks of tanks, the population had drastically declined to less than 100 adult animals in five isolated subpopulations by 2001 (D. Schmidt pers. comm.). It was only after habitat restoration measures had been initiated that the population slowly started recovering. Until today, only two of formerly five reproduction sites within the area are re-used for spawning (D. Schmidt pers. comm.). Thus, the reduced genetic diversity and the inferred population bottleneck of this population are in line with field observations.

The Kalbach population was the only one to exhibit a significant deviation from the Hardy-Weinberg equilibrium, which is indicative of a possible inbreeding effect. Kalbach is the southernmost population of our study and supposed to be the most important population of Yellow-bellied Toads in middle Hesse (Malten & Steiner 2008). The population in Kalbach has benefited from massive support by voluntary field conservationists and is regarded as increasing in size (Malten & Steiner 2008). This information seems to contradict our finding of a significant inbreeding coefficient. As a sampling effect of closely related animals can be excluded, because only adult animals were sampled (see above), the most likely explanation for the significantly elevated F_{IS}-value of the Kalbach population is the occurrence of null alleles at locus 8A. When excluding this locus, the F_{IS}-value of Kalbach will drop to 0.04 and no longer be significant.

Population structure

Landscape fragmentation and the drainage of floodplains and other suitable habitats for semiaquatic animals such as the Yellow-bellied Toad have a major impact on their population structure. In northern Hesse, fragmentation of toad populations due to roads, railways, and urban areas is apparent, and re-establishing the connectivity between most relict populations is considered problematic (Malten & Steiner 2008). Fragmentation of toad populations clearly resulted in reduced population sizes and decreased gene flow between populations and would gain increasing importance if no mitigating measures were undertaken.

With a global F_{ST} = 0.21 and pairwise F_{ST}-values between 0 and 0.42, we found high levels of population differentiation in northern Hesse. In southern Lower Saxony, a global F_{ST} of 0.19 was observed for *B. variegata* (Weihmann et al. 2009). The maximum pairwise F_{ST}-value in northern Hesse (F_{ST} = 0.42) was higher than in other studies (F_{ST} = 0.32 in Lower Saxony and Italy, respectively; Weihmann et al. 2009, Cornetti 2013). The structure analysis supported these findings by emphasizing the isolation of populations at the edge of our study area compared to more central localities.

In contrast to the study by Weihmann et al. (2009), we found indications for a weak isolation-by-distance pattern in northern Hesse, suggesting restricted gene flow between populations. Cornetti (2013) also found a weak correlation between geographic and genetic distances in northern Italian *B. variegata* populations. This is probably a consequence of the generally reduced dispersal ability of the Yellow-bellied Toad (maximum dispersal distances observed range around 2.5 km and possibly 4.5 km; Jehle & Sinsch 2007). However, it is worth mentioning in this context that in years with substantial rainfall and extensive floodings, toads and tadpoles may be washed to more distant localities with effluent water. While no such drifting could as yet be proved for *B. variegata* by field observations, respective observations have been made in *Bufo bufo* (H. Wacker pers. comm.). Schader (1983) likewise assumed that translocation of Yellow-bellied Toads over long distances along the river Rhine flood plain occurred during flooding events. At least in the direction of the main water drainages, gene flow may therefore occasionally occur over very long distances, counteracting the differentiation and establishment of an isolation-by-distance pattern.

To mitigate a further loss of genetic diversity through fragmentation, management efforts should concentrate on stabilizing individual populations as well as reconnecting them. This has worked successfully, e.g., in Melsungen, where conservation measures have focused not only on the local Yellow-bellied Toad population, but also the entire Kehrenbach (stream). The population at Melsungen now has good future prospects and is regarded as a possible source population for reintroduction projects at other sites of the Kehrenbach (Schmidt & Zitzmann 2012).

The only case where no differentiation was observed between populations refers to the localities Obersuhler Aue, Heringen, Bebra, and Blankenheim in the centre of our study area. This result highlights the importance of genetic exchange between populations of the Middle Fulda and Werra river valleys, the two largest river valleys in our study area. While our genetic data suggest a good connectivity of the populations, field observations have not yet proved an exchange of animals between the river valleys of the Fulda and Werra (until now, no specimens have been observed in the Seulingswald at the watershed between Fulda and Werra; H. Wacker pers. comm.). In addition, we have no information on possible anthropogenic translocations of Yellow-bellied Toads in this area. The highway A4, which runs in a north–south direction between the Fulda and Werra, might not act as an absolute barrier to gene flow, because the connecting river valleys are all traversed by sufficiently large bridges and tunnels for streams (H. Wacker pers. comm.). To further facilitate a genetic exchange between Fulda and Werra populations, establishing stepping stone habitats is envisaged, and supportive measures for the largest population of the region in Obersuhler Aue will enhance the emigration of animals to surrounding populations (H. Wacker pers. comm.).

Given the high level of genetic divergence between several of the studied populations, we suggest defining

separate management units for conservation purposes. Populations in Melsungen, Ellenberg, and Kalbach (including Hünfeld) should be considered distinct management units. Another management unit should comprise the highly interconnected populations at Obersuhler Aue, Heringen, Bebra, and Blankenheim. The populations at Breitau, Trimberg and Bad Sooden-Allendorf are interconnected by streams and show little genetic divergence, thus these three populations may also be managed collectively.

Conclusions

The main goal of our study was to provide a first assessment of the genetic situation of the endangered Yellow-bellied Toad in northern Hesse, Germany, and provide guidelines for its conservation. To facilitate interpretability of our results, we compared our data to studies on *B. variegata* in Lower Saxony, Germany (Weihmann et al. 2009), and the Alps in northern Italy (Cornetti 2013).

In northern Hesse, the Yellow-bellied Toad has experienced a dramatic fragmentation of its habitats and reduction in population size due to anthropogenic alterations of habitats (Malten & Steiner 2008). Management measures to counteract these processes have been initiated more than 30 years ago, but no data on the genetic composition of the Yellow-bellied Toad populations in northern Hesse have been available until now.

Our analysis shows that levels of genetic diversity of the Yellow-bellied Toad in northern Hesse are similar to those of other populations at the edge of the distribution range in Lower Saxony and northern Italy. However, the Yellow-bellied Toad has suffered local extinctions and population declines in all three regions in the recent past (DGHT-AG Feldherpetologie und Artenschutz 2014; Barbieri et al. 2004). To evaluate the observed level of genetic diversity in terms of long-term survival, it would be desirable to obtain comparable data from less affected populations, preferably from the centre of the species' distribution range.

Population fragmentation and isolation may result in a loss of genetic variability and, consequently, higher susceptibility to environmental changes in the near future (e.g., Veith & Schmitt 2009). Particularly, populations at the edge of our study area exhibited reduced levels of diversity and a higher degree of isolation. Continuous management efforts should focus on the reconnection of isolated populations and ensure self-sustaining population sizes. Where gene flow between isolated populations cannot be re-established, deliberate translocation of tadpoles between localities may be feasible to maintain gene exchange between populations. To ensure the long-term survival of the Yellow-bellied Toad in northern Hesse, we recommend continuation of a demographic population monitoring supplemented by population genetic analyses that may be more sensitive to recognize a detrimental loss of genetic variability at an early stage.

Acknowledgements

We are most grateful to Detlef Schmidt (Arbeitsgemeinschaft Amphibien- und Reptilienschutz in Hessen e.V., AGAR), Heinrich Wacker (Stadt Rotenburg an der Fulda), and Gabriele and Harald Nicolay (Agri Herp Consult, Hann. Münden), who accompanied us during field trips and provided valuable information on the situation and conservation measures before sampling the populations in northern Hesse took place. Matthias Bendorf and Nico Göbel supported our fieldwork. Our study forms part of the implementation of the species action plan for the Yellow-bellied Toad in the Federal State of Hesse (Malten & Steiner 2008) and was financially supported by the Hessen Forst – Servicezentrum für Forsteinrichtung und Naturschutz (FENA) in Gießen. Entering and sampling permits were kindly issued by Hessen Forst-FENA in 2011 and the Untere Naturschutzbehörde Landkreis Hersfeld Rotenburg and Obere Naturschutzbehörde / Regierungspräsidium Kassel in 2012.

References

Barbieri, F., F. Bernini, F. M. Guarino & A. Venchi (2004): Distribution and status of *Bombina variegata* in Italy. – Italian Journal of Zoology, **1**: 83–90.

Bütehorn, B., C. Geske, S. Jokisch, D. Mahn & M. Weissbecker (2010): Erhaltungszustand von Lebensräumen und Arten der FFH-Richtlinie. – Der hessische Beitrag zum Bericht nach Artikel 17. – Jahrbuch Naturschutz in Hessen, **13**: 12–22.

Bundesamt für Naturschutz (2014): Internethandbuch zu den Arten der FFH-Richtlinie Anhang IV. – Available at http://www.ffh-anhang4.bfn.de/.

Charlesworth, B. (1998): Measures of divergence between populations and the effect of forces that reduce variability. – Molecular Biology and Evolution, **15**: 538–543.

Cornetti, L. (2013): Conservation genetics of the yellow-bellied toad (*Bombina variegata*) and the common lizard (*Zootoca vivipara*) in the Italian Alps. – PhD Thesis, Ferrara. – Available at https://openpub.fmach.it/.

Cornuet, J. M. & G. Luikart (1997): Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. – Genetics, **144**: 2001–2014.

DGHT-AG Feldherpetologie und Artenschutz (2014): Verbreitungsatlas der Amphibien und Reptilien Deutschlands. – Available at http://www.feldherpetologie.de/atlas/.

Earl, D. A. & B. M. vonHoldt (2012): Structure Harvester: a website and program for visualizing Structure output and implementing the Evanno method. – Conservation Genetics Resources, **4**: 359–361.

El Mousadik, A. & R. J. Petit (1996): High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels] endemic to Morocco. – Theoretical and Applied Genetics**, 92**: 832–839.

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. & H. E. L. Lischer (2010): Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. – Molecular Ecology Resources, **10**: 564–567.

Excoffier, L., P. E. Smouse & J. M. Quattro (1992): Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. – Genetics, **131**: 479–491.

Falush, D., M. Stephens & J. K. Pritchard (2003): Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. – Genetics, **164**: 1567–1587.

Fijarczyk, A., N. Nadachowska, S. Hofman, S. Litvinchuk, M. Stuglik, G. Gollmann, L. Choleva, D. Linceanu, T. Vukov, G. Dzukic & J. Szymura (2011): Nuclear and mitochondrial phylogeography of the European fire-bellied toads *Bombina bombina* and *Bombina variegata* supports their independent histories. – Molecular Ecology, **20**: 3381–3398.

Fromhage, L., M. Vences & M. Veith (2004): Testing alternative vicariance scenarios in Western Mediterranean discoglossid frogs. – Molecular Phylogenetics and Evolution, **31**: 308–322.

Geske, C. (2009): Landesweite Artenhilfskonzepte für besonders gefährdete Tier- und Pflanzenarten der FFH-Richtlinie in Hessen. – Naturschutz und Biologische Vielfalt, **69**: 181–190.

Glaubitz, J. C. (2004): Convert: A user-friendly program to reformat diploid genotypic data for commonly used population genetic software packages. – Molecular Ecology Notes, **4**: 309–310.

Gollmann, B. & G. Gollmann (2012): Die Gelbbauchunke – von der Suhle zur Radspur. 2. Aufl. – Laurenti Verlag, Bielefeld.

Goudet, J. (2002): FSTAT, a program to estimate and test gene diversities and fixation indices version 2.9.3.2. – Available at http://www2.unil.ch/popgen/softwares/fstat.htm.

Hauswaldt, J. S., C. Schroder & R. Tiedemann (2007): Nine new tetranucleotide microsatellite markers for the fire-bellied toad (*Bombina bombina*). – Molecular Ecology Notes, **7**: 49–52.

Hedrick, P. W. (1999): Perspective: highly variable loci and their interpretation in evolution and conservation. – Evolution, **53**: 313–318.

Hedrick, P. W. (2005): A standardized genetic differentiation measure. – Evolution, **59**: 1633–1638.

Hewitt, G. M. (2004): Genetic consequences of climatic oscillations in the Quaternary. – Philosophical Transactions of the Royal Society B, **359**: 183–195.

Hofman, S., C. Spolsky, T. Uzzell, D. Cogălniceanu, W. Babik & J. M. Szymura (2007): Phylogeography of the fire-bellied toads *Bombina*: independent Pleistocene histories inferred from mitochondrial genomes. – Molecular Ecology, **16**: 2301–2316.

IUCN (2015): The IUCN red list of threatened species. International Union for the Conservation of Nature. – Available at http://www.iucnredlist.org.

Jehle, R. & U. Sinsch (2007): Wanderleistung und Orientierung von Amphibien: eine Übersicht. – Zeitschrift für Feldherpetologie, **14**: 137–152.

Kühnel, K. D., A. Geiger, H. Laufer, R. Podloucky & M. Schlüpmann (2009): Rote Liste der Lurche. – pp. 257–288 in: Haupt, H., G. Ludwig, H. Gruttke, M. Binot-Hafke, C. Otto & A. Pauly (eds): Rote Liste gefährdeter Tiere, Pflanzen und Pilze Deutschlands. Band 1: Wirbeltiere. – Naturschutz und Biologische Vielfalt, **70**, Landwirtschaftsverlag, Münster.

Lowe, A., S. Harris & P. Ashton (2004): Ecological Genetics. – Blackwell Publishing, Malden, USA.

Luikart, G. & J. M. Cornuet (1998): Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. – Conservation Biology, **12**: 228–237.

Malten, A. & H. Steiner (2008): Artenhilfskonzept Gelbbauchunke (*Bombina variegata*) in Hessen. Aktuelle Verbreitung und Maßnahmenvorschläge. – Hessen-Forst FENA, Gießen. Available at http://www.hessen-forst.de/naturschutz-artenschutz-steckbriefe,-gutachten-und-hilfskonzepte-zu-ffh-arten-2294.html.

Nei, M. (1987): Molecular Evolutionary Genetics. – Columbia University Press, New York.

Neubeck, C. & U. Brauckmann (2014): Gelbbauchunke Nordhessen. Die Gelbbauchunke als Leitart für Pionieramphibien in den Flussauen Nordhessens: Naturschutzgenetik, Populationsökologie und Schutzmaßnahmen. – Universität Kassel. Available at https://www.uni-kassel.de/projekte/fileadmin/datas/projekte/gelbbauchunke/Publikationen/Endbericht_DBU_GbU_NH.pdf.

Nicolay, H. & G. Nicolay (2013): Die Gelbbauchunke (*Bombina variegata*) im Werra-Meißner-Kreis: aktuelle Bestandssituation und Ausblick. – Jahrbuch Naturschutz in Hessen, **14**: 134–140.

Nürnberger, B., S. Hofman, B. Förg-Brey, G. Praetzel, A. Maclean, J. M. Szymura, C. M. Abbott & N. H. Barton (1993): A linkage map for the hybridising toads *Bombina bombina* and *B. variegata* (Anura: Discoglossidae). – Heredity, **91**: 136–142.

Pabijan, M., A. Wandycz, S. Hofman, K. Wecek, M. Piwczynski & J. M. Szymura (2013): Complete mitochondrial genomes resolve phylogenetic relationships within *Bombina* (Anura: Bombinatoridae). – Molecular Phylogenetics and Evolution, **69**: 63–74.

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

Phillipsen, I. C., W. C. Funk, E. A. Hoffman, K. J. Monsen & M. S. Blouin (2011): Comparative analysis of effective population size within and among species: Ranid frogs as a case study. – Evolution, **65**: 2927–2945.

Poschadel, J. R. & D. Möller (2004): A versatile field method for tissue sampling on small reptiles and amphibians, applied to pond turtles, newts, frogs and toads. – Conservation Genetics, **5**: 865–867.

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

Raymond, M. & F. Rousset (1995): Genepop (version 1.2): population genetics software for exact tests and ecumenicism. – Journal of Heredity, **86**: 248–249.

Rice, W. R. (1988): Analyzing tables of statistical tests. – Evolution, **43**: 223–225.

Schader, H. (1983): Die Bedeutung der Rheinauen zwischen Oppenheim und Worms für die Amphiben Rheinhessens. – Berichte aus den Arbeitskreisen der GNOR, AK Rheinhessen, **4/5**: 165–191.

Schlüpmann, M., M. Bussmann, M. Hachtel & U. Haese (2011): Gelbbauchunke – *Bombina variegata*. – pp. 507–542 in: Arbeitskreis Amphibien und Reptilien in Nordrhein-Westfa-

len (ed.): Handbuch der Amphibien und Reptilien Nordrhein-Westfalens. – Laurenti-Verlag, Bielefeld.

Schmeller, D. S. & J. Merilä (2007): Demographic and genetic estimates of effective population and breeding size in the amphibian *Rana temporaria*. – Conservation Biology, **21**: 142–151.

Schmidt, D. & A. Zitzmann (2012): Voruntersuchung möglicher Trittsteinbiotope zur Ausbreitung der Gelbbauchunke im Kehren- und Salmsbachtal (Schwalm-Eder-Kreis; Regierungsbezirk Kassel). – Unpublished report on behalf of the Regierungspräsidium Kassel.

Stuckas, H. & R. Tiedemann (2006): Eight new microsatellite loci for the critically endangered fire-bellied toad *Bombina bombina* and their cross-species applicability among anurans. – Molecular Ecology Notes, **6**: 150–152.

Taberlet, P., L. Fumagalli, A.-G. Wust-Saucy & J.-F. Cosson (1998): Comparative phylogeography and postglacial colonization routes in Europe. – Molecular Ecology, **7**: 453–464.

Tallmon, D. A., M. A. Beaumont & G. H. Luikart (2004): Effective population size estimation using approximate Bayesian computation. – Genetics, **167**: 977–988.

Tallmon, D. A., A. Koyuk, G. H. Luikart & M. A. Beaumont (2008): ONeSAMP: a program to estimate effective population size using approximate Bayesian computation. – Molecular Ecology Resources, **8**: 299–301.

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.

Veith, M. (1996): Verbreitung und Gefährdung der Gelbbauchunke, *Bombina v. variegata* (Linnaeus, 1758) in Rheinland-Pfalz. – Naturschutzreport, **11**: 177–186.

Veith, M. & T. Schmitt (2009): Naturschutzgenetik bei Amphibien: Von der Theorie zur Praxis. – pp. 153–184 in: Hachtel, M., M. Schlüpmann, B. Thiesmeier & K. Weddeling (eds): Methoden der Feldherpetologie. – Zeitschrift für Feldherpetologie, Supplement.

Weihmann, F., R. Podloucky, S. Hauswaldt & H. Prohl (2009): Naturschutzgenetische Untersuchungen von Populationen der Gelbbauchunke (*Bombina v. variegata*) im südlichen Niedersachsen. – Zeitschrift für Feldherpetologie, **16**: 183–200.

Whitlock, M. C. (2011): G'_{ST} and D do not replace F_{ST}. – Molecular Ecology, **20**: 1083–1091.

Wright, S. (1943): Isolation by distance. – Genetics, **28**: 114–138.