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RESEARCH ARTICLE

Gene-flow in the clouds: landscape genetics of a viviparous, montane grassland toad in the tropics

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Abstract Anthropogenic habitat alteration often increases fragmentation and isolation, which decreases population sizes and increases extinction risk for species. Extrinsic threats may be buffered or enhanced by intrinsic factors. Within amphibians, the influence of different environmental and intrinsic factors on the population structure is not yet fully understood. Four factors were found to be important for population connectivity: life history traits, recent (anthropogenic) land use history, habitat, and topography, but the direction of their influence differed between studies. Here, we examine the genetic population structure and interpopulation connectivity within the complete distribution of Nimba toads (*Nimbaphrynoides occidentalis*), a toad from montane tropical West Africa. The Nimba toad is the only known viviparous, matrotrophic (foetuses are nourished during the gestation by their mothers) anuran on Earth. It occurs in three regions, the smallest is situated in disturbed, the largest population in partly disturbed habitat and the third was not yet impacted. We found small, but significant population differentiation, no indication of a recent bottleneck in the smallest population, but an indication of a reduction in population sizes in the more distant past in all three populations and no sex-biased dispersal. Correlations with landscape classifications indicate that high elevations, due to their high humidity levels, are the most important landscape characteristic facilitating dispersal. This underscores desiccation risk as an important landscape characteristic for amphibian population connectivity. We found indication that life-history traits (viviparity), land use history (mining-related activity) and topography (elevation) have an influence on Nimba toad population differentiation and gene-flow.

Keywords Nimba toad · Population genetics · Sex-biased dispersal · Small population size · *Nimbaphrynoides occidentalis* · Desiccation risk

Introduction

Anthropogenic habitat alteration often leads to decreased population size, yet gene-flow through successfully immigrating individuals may reduce detrimental effects of small population sizes (Lacy 1987). Anthropogenic activities often result in decreased connectivity between populations through fragmentation and may thus increase species' isolation and extinction risk (Cornetti et al. 2016). Understanding the population structure, population size, and interpopulation connectivity are crucial for effective conservation actions. As conservation decisions often need to be made within short time, attempts to develop general measures for different taxonomic groups are frequently formulated (Frankham et al. 2014). Albeit amphibians are a highly threatened taxon they are too diverse with respect to life-histories and habitat requirements to enable generalisations for conservation measures. Theoretical assumptions with ambiguous support in practice include the generalisation that amphibians, due

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to their small size and low dispersal abilities, should show strong population differentiation on small geographic scales (Sandberger et al. 2010; Langone et al. 2016); or that in species with high female reproductive investment dispersal should be female-biased (Liebgold et al. 2011; Helfer et al. 2012). Additionally, the interaction between landscape and gene-flow was shown to be species-specific and may differ among ecologically similar (Richardson 2012) and closely related species (Spear and Storfer 2008, 2010). Nevertheless, four characteristics have been identified to have an important but sometimes opposing influence on connectivity of amphibian populations: life history traits (Measey et al. 2007; Savage et al. 2010; Nowakowski et al. 2015), recent (anthropogenic) land use history (Richardson 2012; Cornetti et al. 2016), habitat type (Spear and Storfer 2008, 2010; Nowakowski et al. 2015), and topography (Funk et al. 2005; Spear et al. 2005). Hence, until sufficiently abundant data on population structure, population size, and connectivity is available these characteristics need to be analysed for every focal species to develop sound conservation plans. Studies on species with less well-known traits should add particularly valuable data to determine generalisations for amphibians in the future.

Here, we examine the genetic population structure and interpopulation connectivity within the entire distribution of Nimba toads (*Nimbaphrynoides occidentalis*), a critically endangered toad from montane West Africa. In this matrotrophic viviparous species mothers nourish their offspring during gestation, and after 9 months fully developed toadlets are born (Xavier 1971; Sandberger-Loua et al. 2017). Lifetime reproductive output is very low, with only about 20 young per female (Sandberger-Loua et al. 2017). The species' range is restricted to three regions within the high elevation grasslands of the Nimba Mountains, a 40 km long and 12 km wide mountain ridge (Lamotte 1959; Sandberger-Loua et al. 2016a). This habitat is characterised by the nearly complete absence of open water, including rivers (Leclerc et al. 1955; Sandberger-Loua et al. 2016a), frequent fires during the dry season, and on higher elevations consistent fog during the rainy season (Leclerc et al. 1955). In the past 50 years the anthropogenic influence differed between regions. The two regions at either end of the mountain ridge are (in part) mining concessions (North and Liberia, Fig. 1, Poilecot and Loua 2009). Only the central region of the mountain chain [Richard-Molard (RMolard)] is situated

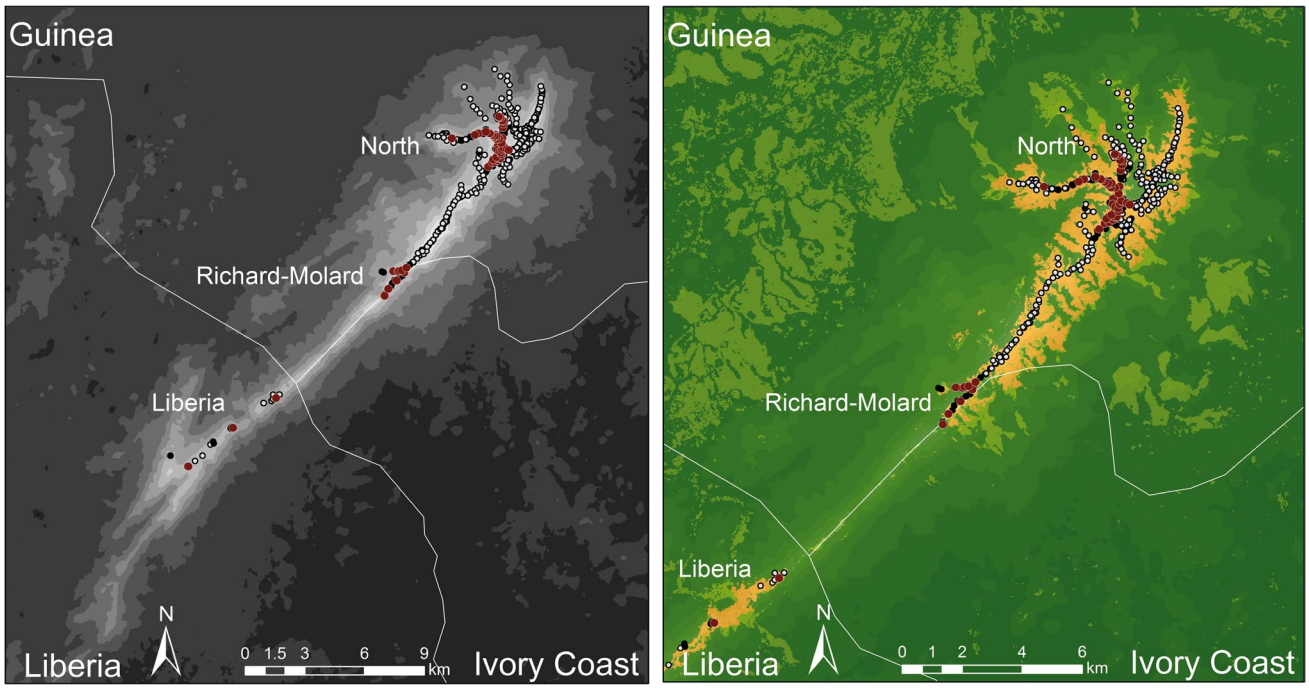

Fig. 1 The West African Nimba Mountains at the border between Guinea, Liberia and Ivory Coast. The left figure illustrates the entire mountain ridge based on the ASTER Global Digital Elevation Model (GDEM, ASTER GDEM which is a product of METI and NASA), the right map shows the different vegetation cover after de Jong et al. (2009) across the Nimba toad's entire range; forests are shown in darker green, savannah in light green and the high elevation grasslands in orange. Sample locations included in this study are shown as red circles; locations examined for toad presence between 2007 and 2017 not included in this study are shown as unfilled white circles (absences) and filled black circles (presences). Please note that circles in Guinea indicate 25 m^2 examined, whereas in Liberia one circle indicates several 100 m^2 searched for toads. Country boundaries are shown as white lines; smaller writings indicate the Nimba toad population names as used throughout the text. (Color figure online)

completely within the World Heritage Site (Poilecot and Loua 2009).

Examining the genetic structure of Nimba toad populations provides valuable insights on how landscape affects this endangered species and will further add a combination of not yet examined landscape and life-history characteristics to the existing studies on the interpopulation connectivity of amphibians. These include a very small geographic range (Hillers et al. 2008; Sandberger-Loua et al. 2016a), high habitat specificity (Lamotte 1959; Sandberger-Loua et al. 2016a), few offspring (Angel and Lamotte 1944; Sandberger-Loua et al. 2016b), assumed poor dispersal abilities and pronounced seasonality (Leclerc et al. 1955). These five factors are also among the traits associated with decline and extinction risk in amphibians (Cooper et al. 2008; Sodhi et al. 2008). Within this study, we examined the influence of life-history traits (viviparity), recent anthropogenic history (mining and mineral exploration), habitat and topography (elevation) on Nimba toad population differentiation and gene-flow. We hypothesised that (i) within the Nimba toads' range three distinct genetic populations exist, corresponding to the three regions of toad occurrences; (ii) the mining activities in Liberia reduced the genetic diversity in that population due to a reduction in effective population size and the disconnection from the two Guinean populations by unfavourable habitat, the mine pit; (iii) toads disperse along the ridges and avoid lower elevations and forests, as they were exclusively recorded in high elevation grasslands; and (iv) Nimba toads show female-biased dispersal, as female reproductive investment is high.

Materials and methods

Study site and study species

The West African Nimba Mountains extend over three countries: Guinea, Liberia, and Ivory Coast (7.71–7.40 N; 8.61–8.32 W). The mountain chain consists of a southwest-northeast oriented undulating main ridge (elevation of the ridge between 1000 and 1650 m asl), which bifurcates in the North (Leclerc et al. 1955). At lower elevations, the mountain chain is dominated by savannah or rainforest (Schnell 1952). Particularly in the South and along ravines in the North rainforest may reach the ridge, but at most high elevations montane grasslands dominate (Fig. 1, Poilecot and Loua 2009). The presence of Nimba toads, chimpanzees, and a rich and endemic fauna and flora led to the declaration of the Guinean (1981) and Ivorian (1982) Nimba Mountains as a World Heritage Site (WHS, UNESCO 2017b). The WHS has been listed as in danger since 1992 (Poilecot and Loua 2009; UNESCO 2017a) and had mainly been kept on this list due to mining-related activities and poor management (UNESCO 2017b). For more details on habitats and conservation history refer to Leclerc et al. (1955), Lamotte (1959), Hillers et al. (2008), Poilecot and Loua (2009), Sandberger-Loua et al. (2016a), UNESCO (2017b).

The Nimba toad (*Nimbaphrynoides occidentalis*) has a unique anuran reproductive mode, being matrotrophic and viviparous. For a review of the toad's reproduction see Sandberger-Loua et al. (2017). Nimba toads are endemic to the high elevation grasslands of the Nimba Mountains (Lamotte 1959; Hillers et al. 2008; Sandberger-Loua et al. 2016a) and assessed as critically endangered (IUCN 2017). Currently, Nimba toads occur at two locations within the high elevation grasslands in Guinea and in the human-derived secondary grasslands in the former mining site in Liberia. Despite extensive field work Nimba toads were never recorded within forests and occur exclusively at elevations above 1200 m asl (Lamotte 1959; Hillers et al. 2008; Sandberger-Loua et al. 2016a). This elevation is characterised by constant fog during the rainy season (Leclerc et al. 1955; Sandberger-Loua et al. 2016a).

Sample collection

Samples for genetic analyses were collected in Guinea in 2008 and 2009, and in Liberia in 2008. The annual Guinean monitoring consisted of 60 sampling locations (5×5 m^2), which were sampled four times in 2008 and twice in 2009 (for a description of methods and sampling site characteristics compare Hillers et al. 2008; Sandberger-Loua et al. 2016a, b). Samples in Liberia were collected across the entire known Liberian area within a single visit in 2008. In total Nimba toads were recorded and sampled at 48 locations. Samples were distributed as evenly over the toad's distribution as possible and we aimed to cover both sexes in equal shares but recorded more females. Samples consisted of a tip of the second toe of adult toads (following procedures described in Grafe et al. 2011) stored in 98% ethanol for DNA analysis. In total, we sampled 600 individuals from all three regions and hence, covered the entire range of Nimba toads (detailed sample sizes are given in Table 1).

Genotyping

DNA for population genetic analyses was extracted using the Roche PCR template preparation kit according to the manufacturer's recommendations. PCRs were performed using a 12.5 µl PCR reaction volume containing $1 \times$ PCR-buffer, 2 mm $MgCl_2$, 160 µm dNTPs, 2.5 µm of each primer (forward primer labelled with fluorescent IR-700 or IR-800 dye by Licor), 0.5 U of Taq DNA polymerase (New England BioLabs), and 1 µl of 1:10 diluted template DNA. Each sample was analysed at eight loci (G07, D03, Nocc4, A09, C05, C10, E06 and F03; for primer details refer to

Table 1 Sample sizes

Region	Year	Individuals		Females		Males		Percent females	
		Comp	Red	Comp	Red	Comp	Red	Comp	Red
Total	All	600	374	341	220	259	154	56.83	58.82
	2008	400	248	234	147	166	101	58.50	59.27
	2009	200	126	107	73	93	53	53.50	57.94
North	All	427	249	251	152	176	97	58.78	61.04
	2008	282	159	170	99	112	60	60.28	62.26
	2009	145	90	81	53	64	37	55.86	58.89
Molard	All	155	113	77	59	78	54	49.68	52.21
	2008	100	77	51	39	49	38	51.00	50.65
	2009	55	36	26	20	29	16	47.27	55.56
Liberia	All	18	12	13	9	5	3	72.22	75.00
	2008	18	12	13	9	5	3	72.22	75.00
	2009	0	0	0	0	0	0		

Given are the sample sizes included within this study on two spatial scales, whole study site and each population for its own (North, Richard Molard (Molard) and Liberia), and on two temporal scales (both years and each year (2008 and 2009)) on its own. For the complete (comp) and the reduced data set (red, excluding full-sibs) are given the number of individuals consisting of the given number of females and males. Percent females, gives the percentage of females on the number of individuals included

Sandberger-Loua et al. 2016b). All loci were amplified on a 2720 Thermal Cycler (Applied Biosystems, version 2.09), G07 and D03 were run at a fixed annealing temperature of 57 °C, and a touchdown program was applied to all other loci (Nocc4, A09, C05, C10, E06 and F03). PCR conditions for the two protocols were as follows: 57 °C: 3 min at 94 °C, 35 cycles of 30 s each at 94, 57 and 72 °C, followed by 20 min at 72 °C; and touchdown: 5 min at 94 °C, 10 cycles with annealing temperature decreasing 0.5 °C per cycle from 63 to 57 °C, with 30 s each at 94 °C, annealing temperature, and at 72 °C, followed by 25 cycles of 30 s each at 94, 55 and 72 °C, followed by 7 min elongation at 72 °C. Allele lengths were analysed with SAGA^{GT} (LICOR). To minimise scoring errors every sample was amplified at least twice for each locus.

Genetic data analyses

We tested the data for linkage equilibrium (LE) using GenPop (Raymond and Rousset 1995; Rousset 2008) and deviations from Hardy–Weinberg equilibrium (HWE) with Arlequin.3.5.22 (Excoffier and Lischer 2010). We tested for presence of loci under selection with BayeScan (Foll and Gaggiotti 2008). We estimated the number of alleles as well as observed and expected heterozygosity per locus, genetic diversity, allelic range (the difference between the maximum and minimum allele size), and the Garza-Williamson statistic (Arlequin.3.5.22, Excoffier and Lischer 2010).

As after Bonferroni correction, not all populations were in HWE and LE, we tested the data-set for scoring errors, stuttering and the presence of null-alleles using Microchecker (van Oosterhout et al. 2004). As spatial and temporal structuring may generate the impression of null-alleles we tested the data on all combination of two spatial scales (all regions together, each region for its own) and two temporal scales (both sampling years together, and each sampling year for its own). Due to these results, we used two datasets, one including all eight loci, the other six loci (excluding E06 and D03). All population genetic analyses were conducted for both numbers of loci.

The presence of full-sibs in a dataset may also lead to allele frequency biases (Moore et al. 2011). We tested for the presence of full-sibs in the dataset using a Bayesian approach (COLONY2, Jones and Wang 2010). The following settings were used: the maternal genotype was unknown, no candidate fathers included, we gave no known population allele frequency, and we used a sib-ship size prior, two runs of medium length and assumed females and males to be monogamous (which is true for 75% of litters, Sandberger-Loua et al. 2016b). Of those individuals determined as full-sibs with a probability > 0.98, we excluded all but a randomly chosen individual in the dataset. In the following, we refer to this dataset as the reduced dataset. All population genetic analyses were conducted for both numbers of individuals.

To examine the relative effects of sample year and sample region we conducted hierarchical analyses of molecular variance (AMOVA) based on R_{ST} implemented in Arlequin.3.5.22 (Excoffier and Lischer 2010). This allowed us to quantify the partitioning of genetic variance within and among the hierarchical levels (between regions, within regions between years and within regions within the same year).

Population structure

We estimated the number of populations K with an individual based Bayesian MCMC approach, including the spatial structure of samples implemented in the Geneland Package (Guillot et al. 2005b; Guedj and Guillot 2011) in R 3.2.4 (R Core Team 2016). We used the spatial F-model (sensu Guillot et al. 2005a), as the presence of full-sibs indicates a correlation of allele frequencies in the considered regions. It was shown that genetic structure at low differentiation levels is better detectable with the correlated than the uncorrelated model (Guillot 2008) and the spatial than the non-spatial model (Guillot et al. 2005a). We introduced an additive noise to the geographic coordinates allowing individuals recorded at the same location to be assigned to different populations (Guillot et al. 2005a). The analysis was repeated for all years together and each year separately to determine the number of populations. For all runs, we used the following parameters: 100,000 iterations, a thinning rate of 100, the maximum rate of Poisson process fixed at the sample size (Guillot et al. 2005a) and a maximum number of nuclei in the Poisson–Voronoi tessellation fixed at three times the sample size. The posterior probability of population membership was calculated for each pixel of the spatial domain and the modal population assignment for each individual was calculated using a burn-in of 200. We allowed the population number to vary between 1 and 10 and for each year and dataset we repeated the analyses five times. If all runs gave the identical best number of K, we ran the respective analysis with the population number K fixed at the best model result and the same parameters as before. Using the determined populations, we calculated pairwise F_{ST} (Arlequin.3.5.22, Excoffier and Lischer 2010), Jost's D values (R package mmod, Winter 2012) and the estimated effective population size (Colony, Jones and Wang 2010), using the same specifications as for the full-sib determination.

Population size and bottlenecks

Following a population bottleneck, the number of alleles decreases faster than the heterozygosity and hence, for a limited number of generations a heterozygosity excess is observed (Cornuet and Luikart 1996). The analyses for recent bottlenecks (BOTTLENECK 1.2.02, Cornuet and Luikart 1996) can be based on the infinite alleles model (IAM), stepwise mutation model (SMM) or a two-phase model (TPM) which is a combination of the two other models (70% SMM). We assumed a recent bottleneck if the sign test and the Wilcoxon test were significantly indicating a heterozygosity excess. Another assumption is that the Garza-Williamson statistic or M-ratio (the ratio of the number of alleles to the range in allele size measured as base pairs) is smaller in populations with a past reduction in population size (Garza and Williamson 2001). Changes in the M-ratio are assumed to be present for more generations than the heterozygosity excess detected by BOTTLENECK (Williamson-Natesan 2005). An M-ratio smaller than the critical value of M = 0.68 (Garza and Williamson 2001) was considered as an indication of a reduction in population size.

Landscape genetics

The idea of landscape genetics is that dispersing individuals try to minimise the cost passing from one population to the other by following more suitable habitat or landscape structures. Using least cost paths, a higher cost is assigned to less permeable areas and the calculated modified geographic distance is more costly and hence "longer" when passing less permeable areas than passing more permeable areas. These least cost paths, in the following, termed modified geographic distance, can be correlated with genetic distance. The hypothesis is that the modified geographic distance reflects the costs for gene-flow in the study species more realistically and thus correlates better with genetic distances among populations, than Euclidean distance alone. In assigning different permeability values to the different landscape classifications several correlations can be compared and the best correlation will reflect the best-included assumption about landscape permeability.

We classified the Nimba Mountains landscape into discrete elevation and habitat categories. Nimba toads were never recorded at elevations below 1200 m asl, are more abundant above 1400 m asl and were never found within forests (Fig. 2, Lamotte 1959; Hillers et al. 2008; Sandberger-Loua et al. 2016a). Hence, we hypothesised that gene-flow occurs on higher elevations and Nimba toads avoid low elevations and forests. To test this hypothesis, we correlated genetic with geographic distances. We tested

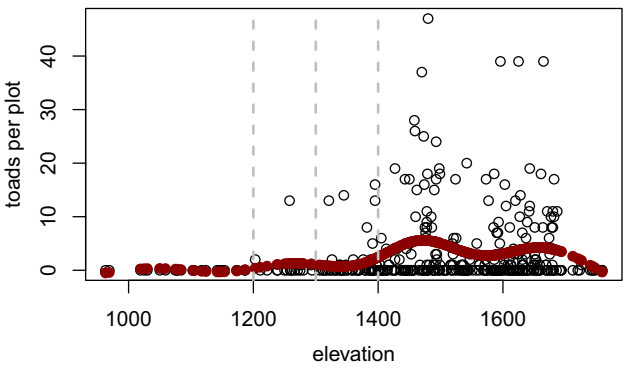

Fig. 2 Dots show toad abundances per 5 × 5 m plots (N = 377 plots) examined 2007 and 2012 (data of Sandberger-Loua et al. 2016a). The red closed circles show the prediction from a generalised additive model (mgcv package, R), based on the field data. The dotted vertical lines indicate 1200, 1300 and 1400 m asl, respectively. (Color figure online)

three hypotheses: (i) genetic distance correlates best with Euclidian distance (isolation by distance, IBD), (ii) genetic distance correlates best assuming that higher elevations have a higher permeability to gene-flow (elevation), (iii) genetic distance correlates best assuming that Nimba toads avoid forests and disperse within savannahs (habitat).

For hypothesis (ii) and (iii) we assigned permeability values to each pixel of a GIS raster layer according to its elevation or habitat category. As it is unknown, at which elevation dispersal is hindered, or which habitat types are important for Nimba toad dispersal, we produced three sets of permeability layers based on the ASTER Global Digital Elevation model retrieved from http://www.ersdac.org; and five sets of GIS permeability layers based on the GIS vegetation classification of de Jong et al. (2009). The three elevation sets differed in the elevation assumed as the boundary between high and low permeability for gene-flow (either 1200, 1300, or 1400 m asl). In the five habitat sets we used different combinations of the following habitat types as classified by de Jong et al. (2009): (i) low elevation forests, (ii) high elevation forests and thickets, (iii) low elevation savannahs, (iv) medium elevation savannah, and (v) high elevation grasslands. We first determined the best correlation between the three sets of least cost paths based on elevation. The correlation with the highest correlation coefficient was compared with the best habitat correlation and the correlation between Euclidian and genetic distance.

We examined the correlation between genetic and geographic distance using Mantel tests between the distance matrices (package ade4, Chessel et al. 2004; Dray et al. 2007) using 999 permutations. All analyses were done in R 3.2.4. Results of the correlations were compared between all modified distances and to the Euclidian distance by favouring the significant models with a higher correlation coefficient. To analyse the spatial effect on landscape connectivity, we analysed all populations together (North, RMolard and Liberia), only the Guinean populations (North and RMolard) and each of the large Guinean populations on their own. Detailed information on methods is given in Supplement 1.

Sex-biased dispersal

We hypothesised that females should disperse, as female reproductive investment is high and hence, inbreeding is costlier for females than males. Additionally, males may gain from being more philopatric than females as they defend territories. We examined the geographic distribution of the determined full-sibs and hypothesised that from a single sibship females should be found more often in more than one region, while males should be found mostly within the same region. Following Goudet et al. (2002), we estimated F_{IS} and F_{ST}, the average relatedness of individuals within a population relative to the whole sample for each sex separately: $r = 2F_{ST}/(1 + F_{IT})$, and the mean and variance of assignment scores (mAIc and vAIc). The AIC describes the probability that an allele is already present in the population, which should be lower for alleles of dispersing individuals. The mean AIC should hence be lower in the dispersing sex. As the dispersing sex includes dispersing and non-dispersing individuals the variance of AICs is larger in the dispersing sex (see FSTAT manual for more details). We expected F_{ST}, r, and mAIc to be lower in females (the assumed dispersing sex), whereas F_{IS} and vAIc should be higher. The significance of sex-linked differences in these statistics was assessed using 10,000 randomizations in the software FSTAT 2.9.3.2 (Goudet et al. 2002).

Results

Genetic analyses

Genotyping for all eight loci was successful for 595 of the 600 individuals, while for five individuals (3 from North and 2 from RMolard) genotyping was successful for five to seven loci only. One individual could not be genotyped at three loci (G07, D03, C05), one at two (G07, F03) and for three individuals one locus each could not be amplified (F03, C10, Nocc4, respectively). In total, we recorded 194 alleles, with on average $24.25 \pm$ sd 6.25 alleles per locus (range 18–35). Average observed (0.86 ± 0.07) and expected heterozygosity (0.89 ± 0.07) was high, as was the average genetic diversity (0.90 ± 0.48) and the allelic range (103.3 ± 18.6 bp; all diversity indices are summarised in Supplement 2). None of the loci showed indication to be under natural selection (compare Supplement 3).

After Bonferroni correction, considering the whole population, four loci were not in HWE, and two loci pairs linked (A09-C10 and D03-C10); but considering each region as population most of the loci were in HWE and only on RMolard one locus pair (C10-C05) was linked (Supplement 4). Within four loci we found significant indications for null-alleles (frequency 0.02 (C10)-0.19 (D03)) and as we had an indication for stuttering in E06, we generated a second dataset excluding D03 and E06. All analyses were calculated with eight and with six loci. As results were congruent we only show the results obtained with the dataset based on eight loci.

To examine the effect of sample year on genetic variability, we estimated the influence of the two sample years with an AMOVA. Only 0.89% of the variance was explained by differences between years, 9.54% between regions and the majority within regions (89.65%, Supplement 5). Excluding Liberia reduced the variance between years to 0.08% and between regions to 1.2% (within regions 98.7%).

149 clusters of full-sibs comprising between two and five individuals were determined by Colony. Sib-ship-size would thus be in accordance with the average number of offspring born per female (nine individuals, Angel and Lamotte 1944; Sandberger-Loua et al. 2016b). 229 individuals were not assigned to a sib-ship. One sib-ship included individuals from all three regions, 44 full-sib-ships included individuals collected in adjacent regions (RMolard and North or RMolard and Liberia) and all other full-sib-ships (104) were comprised of individuals collected within a single region (Supplement 6).

To check the influence of these full-sibs on HWE, LD, Null alleles and the AMOVA, we repeated the analyses with a dataset based on a single randomly chosen individual of each sib-ship only and excluding the five individuals with missing data (n reduced to 374 individuals) to test for effects of family structure. Within this reduced dataset after Bonferroni correction, all locus pairs were in LE, and only Nocc4 deviated from HWE in some populations (Supplement 4). The hierarchical analyses of the genetic variance (AMOVA) gave very similar results for the complete and the reduced dataset, as well as for eight and six loci included (Supplement 5). We calculated every following analysis for the complete and the reduced data set, as results are very similar and sib-ships small, following recommendations of Waples and Anderson (2017) we show only the complete data set.

Population structure

All Geneland analyses estimated the number of $K = 3$ populations, corresponding to the three geographic regions with toad occurrence (Liberia, RMolard and North, compare Supplement 7). Effective population sizes were estimated as 45, 500 and 1000, respectively. In the North we recorded on average 18 and on RMolard (where toads are much more concentrated in a smaller area) even 36 adults per day, whereas in Liberia we recorded on average only 6 adults per day (even so the methods used there were adjusted to record larger numbers of toads than the standardised methods in Guinea). Hence, the small effective population size in Liberia should not derive from lower total sampling effort.

Overall F_{ST} over all temporal and geographic scales was 0.02. The population differentiation was very low but significant ($p < 0.001$) in all pairwise comparisons measured as F_{ST} (for all eight loci in 2008: Liberia-RMolard: 0.08; Liberia-North: 0.08; RMolard-North 0.01 and in 2009 RMolard-North: 0.01). The difference between the Liberian and the two Guinean populations was much larger for Jost's D (in 2008 Liberia-RMolard: 0.45; Liberia-North 0.49; RMolard-North: 0.10; in 2009: RMolard-North: 0.08). Hence, largest differences were found between the Liberian and the two Guinean populations, between the latter two the differentiation was very small.

Genetic indication for small population size in the Liberian population

The analyses of recent reductions in population sizes gave differing results depending on the method used. Using BOTTLENECK, we did not find any indication for a recent bottleneck in the Liberian population with the IAM, TPM, SMM Model for the sign or the Wilcoxon test. For the Wilcoxon test, within RMolard and North populations in 2008 and 2009, we found indication of significant heterozygote excess for the IAM Model, but not in the Wilcoxon tests for one of the other models (TPM, SMM) and for the sign test only in the North 2008 as well for the IAM model (compare Supplement 8). Hence, in contrast to our expectation there were no indications for a recent bottleneck for the highly impacted Liberian population, but rather for the large Guinean populations. Stronger supported indication of a recent reduction in effective population size was observed in the more impacted Northern population, where the anthropogenic alteration was particularly high in 2008. Assuming a critical M-ratio of $M = 0.68$ (Garza and Williamson 2001), the Garza-Williamson index showed an indication of a recent reduction in population size in all populations. The Garza-Williamson statistic ranged between $M = 0.18$ and 0.25. The minimum M-ratio was recorded in Liberia and the maximum at RMolard (Supplement 2). The very low M-ratio values may indicate that population sizes are generally small in Nimba toad populations, or that in the more distant past, the whole mountain chain was impacted, resulting in a past bottleneck.

Landscape genetics

We identified elevation above 1400 m asl as the most important factor explaining genetic population structure, while habitat type played only a lesser role. On all three geographic scales, the correlation with the highest permeability for elevations above 1400 m asl had the highest correlation coefficient (r value) and was significant after Bonferroni correction (in the following termed "best elevation model", Fig. 3). Within the elevation sets the correlations giving higher permeability to the higher elevations were better than giving higher permeability to lower elevations. The best vegetation correlation was within the vegetation set giving highest permeability to high elevation grasslands, medium permeability to low and mid-elevation savannahs and lowest permeability to low and high elevation forests (Fig. 3). The best permeability layers for the best elevation and best vegetation set and Euclidian distance are given in Table 2. More results are summarised in Supplement 1.

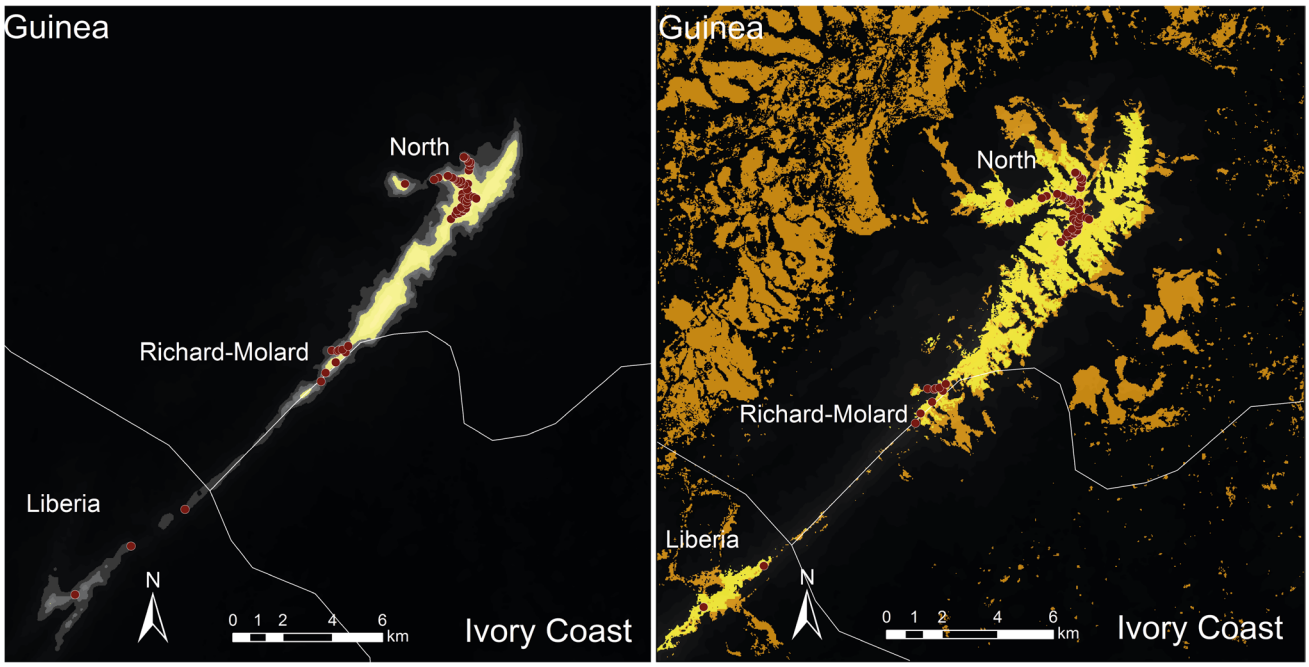

Fig. 3 Visualisation of the best elevation and the best vegetation model. Left: the area most permeable to Nimba toad gene-flow for the best elevation model with the elevational boundary at 1400 m asl is shown in yellow. The two grey-scale areas show the areas above 1300 m asl, lighter grey and yellow, and above 1200 m asl, darker grey, lighter grey and yellow. The map on the right shows the three permeability categories within the best vegetation model. The most permeable vegetation cover were the high elevation grasslands (yellow), medium permeability were given to low and mid-elevation savannahs (orange) and lowest permeability to low and high elevation forests (black). The red circles depict the capture locations of Nimba toads included in this study. (Color figure online)

Table 2 Landscape genetics

Region	Euclidian		Elevation 1400		Vegetation set 4	
	r	p	r	p	r	p
All	0.376	0.007	**0.504**	0.001	0.50	0.005
GN	0.021	ns	**0.519**	0.001	0.04	ns
North	0.383	0.001	**0.616**	0.001	0.39	0.001
Molard	0.625	0.052	**0.849**	0.024	0.64	ns

Given are the correlation coefficient (r) and the p value (p) for each correlation for Euclidean distance, the best elevation correlation (elevation 1400, with permeability of 20 for elevation above 1400 m asl, and permeability of 1 for elevation below 1400 m asl), and the best vegetation model (vegetation set 4, with permeability of 20 for high elevation grasslands, 10 for medium and low elevation savannahs, 1 for low and high elevation forests). Region gives the geographical scale. Highest correlation coefficients r, indicating the best correlation for each geographical scale are shown in bold. Data shown is for the complete data set and 8 loci

All complete distribution of Nimba toads (North, RMolard and Liberia), *GN* distribution within Guinea (North and RMolard), *North* the northern population by its own, *RMolard* the Richard-Molard population by its own

Sex-biased dispersal

We had hypothesised that females are dispersing and hence, should have lower mean assignment scores (mAIc), F_{ST} and relatedness index (r) and higher variance of assignment scores (vAIc) and F_{IS} values. This hypothesis was not supported by our non-significant randomisation tests (compare Supplement 6). In addition, the analyses of the sex-specific distribution of the 149 full sib clusters over regions did not indicate sex-biased dispersal (Supplement 6). We recorded 74% of full-sibs within the same region, and 9% of individuals at the same location, but of

those only 1.6% at the same time and only one pair (0.3%) within the mating season.

Discussion

In general, it is assumed that amphibians are highly philopatric and poor dispersers (Smith and Green 2005), and that life-history traits, as for example body size and reproductive mode, are important for population structure (Measey et al. 2007; Wollenberg-Valero 2015). We found the global Nimba toad populations to be structured in three distinct subpopulations. These corresponded to the three regions of their occurrence, the inter-population distance being about 7 km. This distance is smaller than the estimated 10 km for anuran population differentiation (Smith and Green 2005). Yet, Nimba toads are particularly small (~20 mm snout-vent length: SVL, Sandberger-Loua et al. 2017). Small body size decreases dispersal abilities and results in stronger population differentiation (Paz et al. 2015; Wollenberg-Valero 2015, but see; Fouquet et al. 2015; Langone et al. 2016). Additionally, Nimba toads may frequently die during dispersal. A dispersing toad trapped between populations at the beginning of the dry season quite likely dies from desiccation or fire due to the assumed absence of protective dry season dormancy sites (Sandberger-Loua et al. 2016a). Hence, the distance among regions, the small body size and the harsh conditions during the dry season may result in the highly significant population differentiation. Reproductive mode was shown to influence population structure in amphibians (Measey et al. 2007; Fouquet et al. 2015; Mims et al. 2015; Paz et al. 2015). Species showing reproduction independent of open water, e.g. due to direct development (Measey et al. 2007) may show low levels of population differentiation. Nimba toads are viviparous and lack any aquatic larval stage. The recorded significant but low levels of population differentiation are in accordance with the hypothesis that amphibians with water-independent development show higher levels of gene flow. Thus, the population differentiation found in this study may be influenced by factors that should result in significant population differentiation, e.g. small body size and inter-population distance, while the independence of breeding ponds may counteract the differentiation processes.

Habitat alteration may lead to a decrease in population size and subsequently result in a genetic bottleneck (Richter et al. 2009). However, for the small Liberian subpopulation, we found no indication for a recent bottleneck based on heterozygosity excess. In contrast, in all subpopulations the M-ratios indicate a reduction in population size in the past. Changes in the M-ratio are assumed to be present for more generations than heterozygosity excess (Williamson-Natesan 2005). Prior to mining, the Liberian Nimba mountains were forested (Leclerc et al. 1955) and in the 1960s Nimba toads were recorded in "several populations" (Xavier 1978). This supports the assumption that in Liberia the subpopulation size was always small and fluctuating, possibly masking heterozygosity excess (Peery et al. 2012). Similarly, it was hypothesised that the Guinean mountains were predominantly forested and only due to anthropogenically induced fires, the large area of high elevation grasslands was formed (Schnell 1952). According to this hypothesis, the Guinean Nimba toads could have been restricted to forest clearings and thus would have had small and fluctuating subpopulations. Starting in 1955, the Liberian Nimba Mountains were explored and between 1963 and 1989 mined for iron ore (Lubke and Branch 2000). Since 1957, the Guinean and Ivorian Nimba mountains were explored for mineral extraction (Camara 2001, J. Suter personal communication). In Liberia from the 1960s to 2007 toad abundance decreased from >2 toads per m^2 (Xavier 1978) to an average of 0.03 toads per m^2 (own unpublished data). In Guinea abundances changed from 3.5 (Xavier 1971) to 0.77 and 0.67 toads per m^2 for the RMolard to 0.69 and 0.48 toads per m^2 in the North in 1991 and now, respectively (Bangoura 1993, own unpublished data). Hence, all three subpopulations indicate a reduction in population size and low M-ratios, but determining the cause of this reduction is difficult. First, the reduction in population size indicated by M-ratios may be detectable for a long time (Cornetti et al. 2016) and an event in the unknown past (before the toad's description and mining activities) may have resulted in the observed pattern. Second, no data on population size exist between the 1960s (Lamotte 1959; Xavier 1971) and 1991 (Bangoura 1993; Hillers et al. 2008; Sandberger-Loua et al. 2016a) and we, therefore, do not know whether population sizes decreased steadily or fluctuated. Third, if mineral exploration has caused the reduction in population size, we would expect that population sizes differ significantly between the North and RMolard, which is not the case. Hence, currently we can only assume that the impacts of mining-related activities are sufficient to explain the observed M-ratios in the subpopulations.

In vertebrate species with high male resource defence competition and high female reproductive investment, female-biased dispersal, and in species with high competition over mates, male dispersal is more likely (Greenwood 1980; Helfer et al. 2012). Some amphibian species follow this rule (e.g. Austin et al. 2003), others do not (e.g. Lampert et al. 2003; Helfer et al. 2012). We expected that due to their high reproductive investment Nimba toad females are more likely to disperse and that territorial males, which show high levels of male–male resource defence competition over these territories (Sandberger-Loua et al. 2016b), are more philopatric. However, we did not find sex-biased dispersal. Other studies on amphibians with water independent

reproduction and high female investment likewise revealed no indication of female-biased dispersal (e.g. Helfer et al. 2012). We hypothesise first, that at least in Guinea, inbreeding avoidance for females is not yet an important factor, and second, that philopatric males have no reproductive advantage over dispersing males. First, females may not need to avoid inbreeding due to three observations: small litter sizes, currently quite high toad densities, and density-dependent factors, such as social stress. Females and small males may avoid being harassed by large males (Sandberger-Loua et al. 2016b) by moving varying distances, irrespective of sex and hence avoiding close proximity between close relatives. Second, male territories need to be established anew every year, giving immigrants and philopatric males an equal chance to successfully establish territories before the breeding season. Due to these reasons, we assume close relatives are unlikely to meet and mate and inbreeding is not yet a strong evolutionary force. Hence, it seems that in Nimba toads neither the mating nor the reproductive system promotes dispersal in either of the sexes.

For montane amphibians topography, mainly elevation (Funk et al. 2005; Kershenbaum et al. 2014) and slope (Kershenbaum et al. 2014; Mims et al. 2015), or abiotic factors, mainly desiccation risk (Peterman et al. 2014; Emel and Storfer 2015), were shown to have an important influence on population structure and connectivity. In accordance with these findings, our best model (1400 m asl elevation model) includes topography (elevation) and presumably desiccation risk. Whereas desiccation risk is often associated with different habitat types (e.g. Nowakowski et al. 2015), at Nimba it is more likely connected to elevation, with higher elevations being more humid than lower elevations (Lamotte 1959; Sandberger-Loua et al. 2016a). This may explain the lower importance of habitat type than elevation for Nimba toad gene flow. Despite the large importance of high elevations, lower elevations (and forests) were no complete barrier to gene flow as our results show that Nimba toads can traverse lower elevations and forests in the more humid southern end of their range. Hence, our results underline the importance of corridors with low desiccation risk, either given by habitat type or topography, to allow gene-flow among amphibian populations.

For the Nimba toad's conservation, our results have several implications. The three subpopulations are distinct and all indicate past reductions in population sizes. This emphasises the importance that the persistence of favourable environmental conditions in each of the regions needs to be ascertained. The smallest and anthropogenically most impacted Liberian subpopulation shows lower genetic diversity than the larger subpopulations, decreasing this subpopulation's evolutionary potential and increasing its extinction risk. Hence, conservation efforts for this region are of utmost importance. Nevertheless, no heterozygosity excess due to a recent bottleneck was found and we have an indication that negative effects for genetic diversity and evolutionary potential are buffered by immigrants from the RMolard subpopulation. Among the two Guinean subpopulations level of gene-flow is even higher, demonstrating the importance of dispersal for the current population structure. We found that high elevations and high elevation grasslands, presumably due to their lower desiccation risk, are most important, but that other elevations are no complete barrier for gene-flow. This highlights the importance of the elevated humidity levels on the whole mountain chain for Nimba toad connectivity and should not change in the future due to anthropogenic activities. Hence, conservation efforts should not only focus on decreased human impact within regions with Nimba toad presence but should include the landscape and particularly the current high elevation grasslands between these regions, with particular emphasis on elevational humidity levels.

Conclusion

With this study, we examined a tropical anuran species with a unique reproductive mode, occurring in a rare habitat. We found that life-history traits (viviparity), recent anthropogenic history (mineral exploration), and topography (elevation) may have an influence on Nimba toad population differentiation and gene-flow. Our results are in accordance with the hypothesis that amphibian species with a reproductive mode allowing for a more uniform distribution of individuals, leads to low levels of population differentiation. We assumed that the recent history of mining-related activities of the full Nimba Mountains had impacts on the genetic properties of the Nimba toad subpopulations by disrupting suitable habitat, but that these negative effects were buffered by gene-flow. We found that the very humid high elevations promoted gene-flow among populations, emphasising that desiccation risk is an important landscape characteristic for amphibian population connectivity. Additionally, this emphasises that for the Nimba toad's conservation the humidity levels on the whole mountain chain need to be maintained.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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