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Conservation genetics of a mirrored population of the European tree frog (*Hyla arborea*)

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Abstract. Population decline and local extinction of amphibian populations have increased in the last decades. The European tree frog (*Hyla arborea*) is particularly endangered in its northern distribution range. One of the suggested conservation strategies for threatened amphibians is resettlement. The Ökologische Schutzstation Steinhuder Meer e.V. (ÖSSM) started a resettlement project in 2008 for the European tree frog in Lower Saxony and North Rhine-Westphalia, Germany. Hereby, individuals were translocated from a donor population to a location approximately 40 km distant, establishing a mirrored population. The objective of this study was to monitor genetic diversity of these two populations and genetic exchange with a nearby located population at Lake Steinhuder Meer. Therefore, a total of 91 individuals were analysed at 12 species-specific nuclear markers (microsatellite loci). The genetic diversities of the three populations were almost similar but the mirrored population (H_e = 0.66) exhibited a small reduction compared to its donor population (H_e = 0.72). Significant indications for a recent bottleneck were detected for the donor and mirrored population. However, the mirrored population seems to have recovered from founder effects, since the actual number of calling males surveyed at this site since establishment reveals a stable and steadily growing population. F_{ST} values and D_{est} values showed significant differentiation between all sites with a global F_{ST} of 0.08. Likewise, results of a Bayesian clustering analysis indicated the existence of three genetic clusters. The software Geneclass2 assigned nearly half of the individuals of the mirrored population to its source population. Furthermore, the analysis suggested recent migration between the mirrored population and Lake Steinhuder Meer. Compared to other *Hyla* populations the genetic diversity was high at all localities and population sizes seem to have increased. We conclude from our study that resettlement projects can be efficient measures to counteract amphibian population decline when supported by population genetic analyses.

Key words. Amphibia, Anura, *Hyla arborea*, conservation, translocation, mirrored population, genetic diversity, population structure, bottleneck.

Introduction

Amphibian populations have suffered severe declines due to human interventions in nature. Currently, they are considered as the most endangered vertebrate group (Stuart et al. 2004), and are at risk of mass extinction (Wake & Vredenburg 2008). Some suggested main threats to amphibians are water pollution, atmospheric pollution, UV-radiation, invasive species, diseases (Alford & Richards 1999, Beebee & Griffiths 2005), infections such as the chytrid fungus *Batrachochytrium dendrobatidis* (Daszak et al. 2000, Stuart et al. 2004, Skerrat et al. 2007) as well as loss of spawning ponds, habitat destruction and fragmentation (Weissmair 1996, Cushman 2006). The latter could lead to the loss of connectivity among and within populations and result in isolation. Isolation, in turn, favours inbreeding and genetic drift, which cause loss of genetic variation and increase the risk of extinction (Frankham & Ralls 1998).

Molecular analyses have gained importance in conservation management (Pearse & Crandall 2004) and are frequently used to investigate the consequences of habitat fragmentation on amphibian populations (e.g., Palo et al. 2004, Beebee & Griffiths 2005, Krug & Pröhl 2013). Hereby, several studies uncovered low genetic diversity for populations affected by fragmentation (e.g., Andersen et al. 2004, Arens et al. 2006). For example, *Bufo calamita* (Allentoft et al. 2009) and *Plethodon cinereus* (Noël et al. 2007) were found to suffer from loss of genetic diversity caused by the separation of formerly linked meta-populations. Those findings underline the importance of habitat connectivity and maintenance of genetic diversity, as high diversity supports tolerance and adaptability to current changing environments (Booy et al. 2000). Recommended means to minimise genetic depletion are to increase connectivity between populations (e.g., Angelone & Holderegger 2009, Le Lay et al. 2015), habitat protection,

and resettlement projects such as repatriation, relocation, and translocation (Dodd & Seigel 1991, Storfer 1999). In the last decades translocation projects have become an emerging conservation method and they have been conducted for several endangered animal classes such as mammals (e.g., Smith & Clark 1994: black bears; Moehrenschlager & Macdonald 2003: swift foxes), reptiles (e.g., Reinert & Rupert 1999: timber rattlesnakes; Tuberville et al. 2005: gopher tortoises; Field et al. 2007: desert tortoises), and amphibians (e.g., Zvirgzds et al. 1995, Angelone & Holderegger 2009, Dubey et al. 2009, Broquet et al. 2010: European tree frog; Schröder et al. 2012: fire-bellied toad).

The European tree frog is an endangered amphibian species in Lower Saxony and it is categorised as vulnerable in Germany (Podloucky & Fischer 2013). Among several species conservation activities, the Ökologische Schutzstation Steinhuder Meer e.V. (ÖSSM) arranged a resettlement project in 2008 in the district of Schaumburg, Lower Saxony, to re-establish a population in its earlier distribution range. Hereby, an autochthonous population located at Minderheide (MIH) served as the source population and tadpoles were translocated to Sachsenhäger Niederung (SAN) to establish a mirrored population. The tadpoles stemmed from waters that would have dried up before their metamorphosis. The translocation project succeeded in the formation of a stable population. However, since only a part of the gene pool (about 2,000 tadpoles out of 30 clutches) of the source population has been introduced to the new location, it remained unclear if the population is genetically diverse enough to maintain fitness and evolutionary potential. Therefore, the objective of this study was to investigate whether the mirrored population has genetically changed in comparison to its source population. Particularly, we used microsatellite markers to determine genetic diversity and genetic differentiation of the populations. We further examined whether the mirrored population suffered from a recent bottleneck due to founder effects. As a second goal, we analysed the potential to establish migration and gene flow to a nearby continuously growing population at Lake Steinhuder Meer (STM) which is assumed to be connected to SAN through stepping stone habitats.

Materials and methods

Study sites

Study sites (Fig. 1) were located in North Rhine-Westphalia (MIH) and in Lower Saxony (SAN, STM). MIH, a former military area and now used as pasturage, is a nature reserve in the district of Minden-Lübbecke. It unites several structural and vegetational components such as grassland, natural waters, single shrubs, trees and hedgerows in an area of about 31 ha (Bezirksregierung Detmold 2008). SAN with an area of 350 ha with pastures, crop fields, and wooded parts belongs to the district of Schaumburg. The nature reserve Meerbruchswiesen at the Western side of STM has an area of about 1020 ha and is characterized by wetlands, small ditches of different size, smaller groves, and hedges (Bezirksregierung Hannover 1998). It is situated in parts of the districts of Hannover, Nienburg (Weser), and Schaumburg.

Resettlement project at SAN in 2008

Before 2007 no suitable spawning-ponds existed at SAN and tree frogs had been absent for decades (Brandt 2007, personal observation). Because this area provides optimal conditions for conservation strategies (extensive land use, prohibition of insecticides), several ponds were built for the conservation of aquatic amphibian species such as the crested newt (*Triturus cristatus*), the common frog (*Rana temporaria*) and the European tree frog (*Hyla arborea*). The first four ponds were built in autumn 2007, followed by another six ponds in autumn 2009 and four new ponds during the winter season in 2013/14 (ÖSSM 2009, unpubl. data).

The autochthonous population at MIH was chosen as source population for the resettlement project due to various reasons. MIH was the spatially closest population to SAN which might provide similar ecological conditions and selective pressures, therefore, inducing similar traits advantageous for the original and the new habitat. Furthermore, the population size allowed a removal of tadpoles without harming the population. The tadpoles had a high mortality risk, because they lived in ponds that would have desiccated within the next few days (Brandt 2008, personal observation). In June 2008, a total of 2000 tadpoles (~ six week old) collected from three adjacent ponds at MIH were released into three contiguous new ponds at SAN (1,000, 750 and 250 tadpoles) (ÖSSM 2009, unpubl. data). No other introductions were performed within the area. In the following year, 2009, the first male tree frogs were observed calling at the three releasing ponds. Chorus sizes were evaluated annually by one of the authors (TB) on the basis of different categories (1–2, 3–5, 6–10, 11–20, 21–50, 51–100, 101–200 calling males) (modified from Manzke & Podloucky 1995). Each chorus was assigned to one category and the mean numbers of calling males at each pond were summarised to calculate the total number of calling males. Evaluations were based on data of the night with the highest amount of calling males. The amount of calling males increased steadily from 2008 to 2015 and resulted in 341 calling males in ten distinct ponds in 2015 (Fig. 2).

Frog sampling

Since the European tree frog usually migrates less than four kilometres (Stumpel & Hanekamp 1985, Fog 1993), clusters of ponds nearer than four kilometres are defined as one geographic population. During several nights of the breeding season from April to May 2015, buccal swabs (Pidancier et al. 2003, Broquet et al. 2007) from 91 frogs were collected from MIH (28 individuals), SAN (31 individuals),

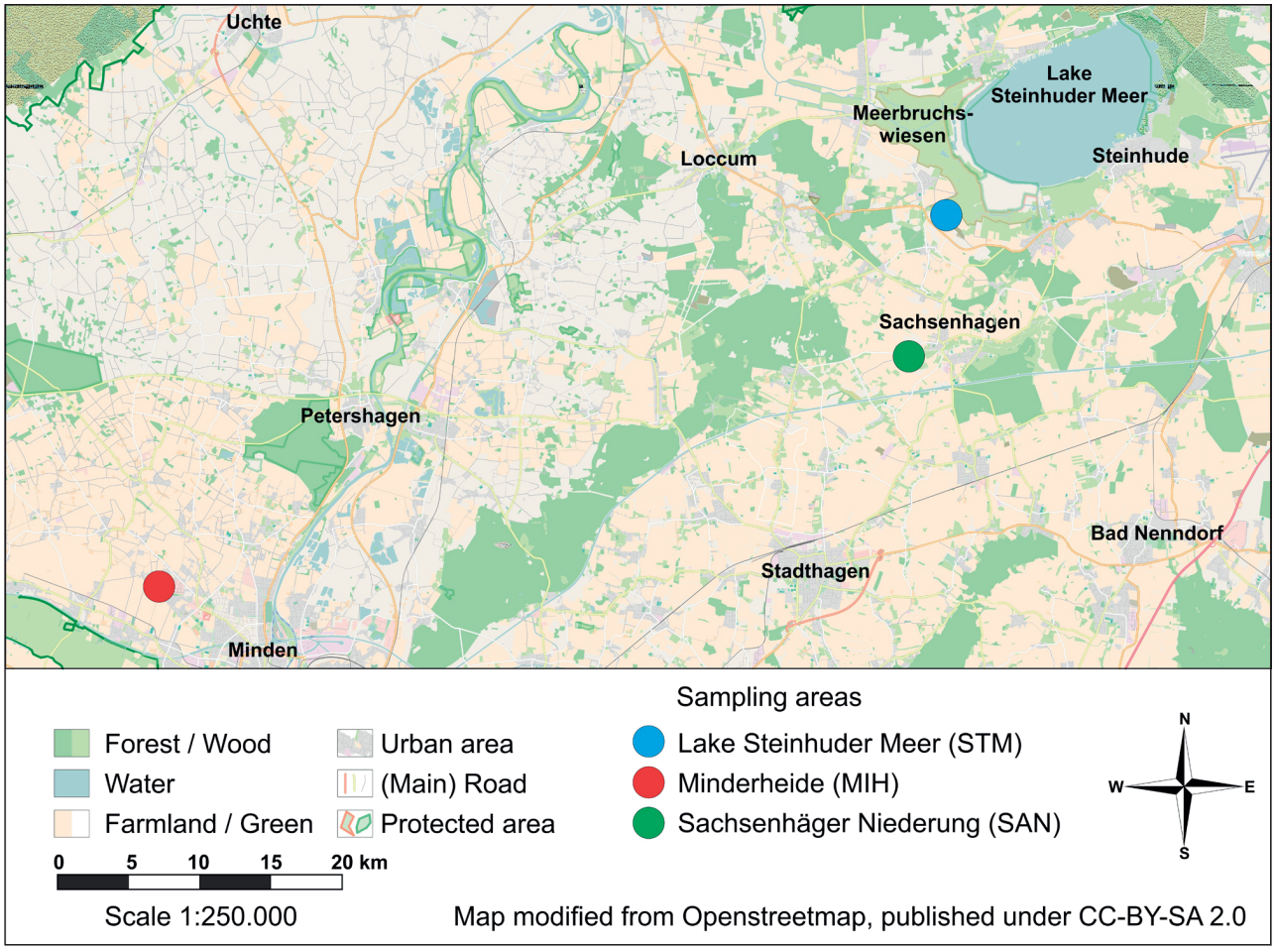

Figure 1. Map of sampling areas in Lower Saxony and North Rhine-Westphalia. The circles represent the sampling areas. The coloured areas represent different types of landscapes as explained in the legend.

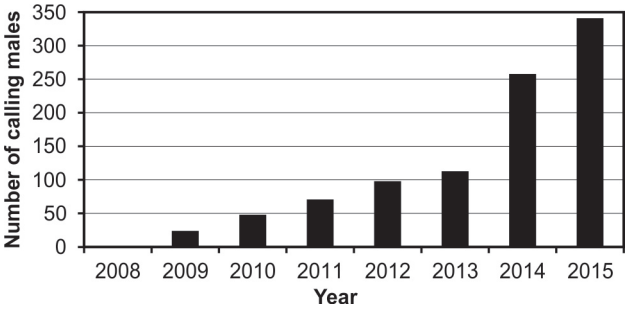

Figure 2. Number of calling males from 2008 until 2015 in the mirrored population at Sachsenhäger Niederung (SAN).

and STM (32 individuals). For representative analyses on the three different geographic populations, the whole area of each sample site was covered by sampling different ponds spread all over the site. Most of the sampled frogs were calling males, but when found females and young adults were also sampled. The saliva samples were air-dried, and kept in Eppendorf tubes at -20°C. After sampling, all frogs were released to the place of capture. Between different samples, instruments were disinfected with 96% ethanol.

Laboratory work

DNA extraction from buccal swabs was performed with the Invisorb® Spin Swab Kit (Stratec Molecular GmbH). The extracted DNA was diluted with ddH_2o in a ratio of 1:5, delivering working stocks for further proceedings, and stored at -20°C. A total of 12 species-specific microsatellite loci (see Appendix 1) were amplified via PCR in specific PCR-programs (see Appendix 2) with different annealing temperatures for each primer according to Arens et al. (2000) and Berset-Brändli et al. (2008). The PCR success was assessed via gel electrophoresis and PCR-products were genotyped with the ABI 3500 genetic analyser (Applied Biosystems, Woolston Warrington) and the ROX Size Standard (Applied Biosystems, Woolston Warrington). Genotyping results were analysed with the program Genemapper version 5 (Life Technologies).

Statistical tests

All loci were tested for the presence of null alleles with Microchecker v. 2.2.3 (Van Oosterhout et al. 2004). The program Fstat v. 2.9.3.2 (Goudet 1995) was used to calculate average allelic richness (based on a minimum reference sample size of 26 diploid individuals), gene diversity and to test for linkage disequilibrium of all pairs of loci. Number and frequencies of private alleles were assessed with GenAlEx 5.6 (Peakall & Smouse 2012). Microsatellite data were analysed for expected and observed heterozygosity (H_e, H_o) as well as for deviation from Hardy-Weinberg equilibrium with the program Arlequin v. 3.5.2.2 (Excoffier et al. 2005). Arlequin was further used to calculate pairwise F_{ST} values (Weir & Cockerham 1984) and to determine the global F_{ST} value by hierarchical molecular analysis of variance (AMOVA). Allele frequencies and D_{est} values (estimation of actual differentiation) were calculated with the package DEMEtics in RStudio (Gerlach et al. 2010). The genetic population structure was examined with the program Structure v. 2.3.4 (Pritchard et al. 2000, Falush et al. 2003). Analyses were performed based on the admixture model for K = 1 to K = 5 with 500,000 iterations each after a burn-in period of 100,000. Twenty runs were performed per each K. The estimated number of true clusters ΔK was calculated as described in Evanno et al. (2005). A genetic assignment test and a test for first generation migrants was conducted with Geneclass2 (Piry et al. 2004) based on Bayesian approaches (Rannala & Mountain 1997) and a simulation algorithm according to Paetkau et al. (2004). Thereby, a number of 10,000 simulated individuals and a type I error (alpha) of 0.01 were set. Heterozygote excess, as evidence for a recent genetic bottleneck, was examined with the program Bottleneck v.1.2.02 (Cornuet & Luikart 1996) with 10,000 iterations assuming TPM (two-phased model of mutation). Since the choice of mutation model influences the results of Bottleneck, three different input values were chosen 1) based on recommendations by Piry et al. (1999) with 95% SSM (single step mutation) in TPM and a variance of 12 (Krug & Pröhl 2013), 2) with 80% SSM in TPM and a variance of 12 (Krug & Pröhl 2013) and 3) with 70% SSM in TPM and a variance of 30 used by Arens et al. (2006). As implemented in Bottleneck, significant heterozygote excess was tested by applying two statistical tests: the Wilcoxon signed-rank test and a 'mode shift' indicator, being able to differentiate between bottlenecked and stable populations (Piry et al. 1999). For all multiple comparisons sequential Bonferroni corrections were applied (Rice 1989).

Results

Data quality

All loci were polymorphic for each sample site except for locus Ha B12 which was monomorphic at MIH and SAN. After applying Bonferroni corrections, locus WHA1-60 showed a significant excess of heterozygotes, whereas the other eleven loci did not deviate from Hardy Weinberg equilibrium. Allelic richness ranged from 1.3 (Ha-B12) to 7.2 alleles per locus (WHA1-140§) with a mean of 4.7 and exhibited its maximum value for STM. Allele frequencies varied between 0.005 and 0.929. The number of alleles ranged from 2 (Ha B12) to 9 (WHA1-67§, WHA1-140§) alleles per locus (see Appendix 1). There was no significant linkage disequilibrium at any pair of loci after applying sequential Bonferroni corrections. Signs of null alleles were uncovered in locus WHA1-20§. As a null allele for this locus was found at a single sample site only (MIH), no adjustment for null alleles was performed.

Table 1. Summary statistics for genetic diversity at 12 microsatellite loci surveyed in *H. arborea*. MIH – Minderheide; SAN – Sachsenhäger Niederung; STM – Lake Steinhuder Meer. n – sample size; H_o – observed heterozygosity; H_e – expected heterozygosity. Standard deviations for H_o and H_e are given in parentheses.

Sample site	n	Gene diversity	H_o	H_e	Allelic richness
MIH	28	0.66	0.72 (±0.168)	0.72 (±0.123)	4.77
SAN	31	0.56	0.66 (±0.130)	0.62 (±0.159)	3.81
STM	32	0.64	0.65 (±0.099)	0.65 (±0.044)	5.41
Average		0.62	0.68	0.66	4.70

Genetic diversity

The three populations differed only slightly in genetic diversity (Table 1). Gene diversity and expected heterozygosity were lowest for SAN and highest for MIH. Observed heterozygosity over all loci exhibited its minimum for the sample site STM and its maximum for MIH.

Genetic differentiation

The degree of genetic differentiation between study populations was determined by calculating pairwise and global F_{ST} and pairwise D_{est} values. All pairwise F_{ST} and D_{est} values were significantly different from 0 (Table 2). The AMOVA analysis displayed a significant genetic population structure (global $F_{ST} = 0.08$, $p < 0.001$) with 8.1% of genetic variance found among populations and 91.9% within them. All populations revealed loci with private alleles. Most of the private alleles were found in the population at STM, whereas the populations at MIH and SAN exhibited similar allele compositions (see Appendix 3).

Population structure and genetic assignment

The Structure analysis in combination with the ΔK calculations supported the existence of three genetic different clusters (Fig. 3, Fig. 4) which correspond to the three geo-

Table 2. Summary statistics for genetic differentiation at 12 loci surveyed in *H. arborea*. Pairwise F_{ST} values are shown below diagonal, pairwise D_{est} values are highlighted and shown above diagonal. Significant values after Bonferroni correction are in bolds. MIH – Minderheide; SAN – Sachsenhäger Niederung; STM – Lake Steinhuder Meer.

	MIH	SAN	STM
MIH		0.098	0.166
SAN	0.050		0.226
STM	0.071	0.115	

graphic populations. Hereby, MIH (green) as well as SAN (red) showed genetic components of the other population. The genetic proportion of STM (blue) was low in both clusters. In turn, there was a considerable genetic influence of both populations within the STM cluster, especially from MIH and to a lesser degree from SAN. The likelihood of the outcome does not increase with greater K.

Assignment of individuals to populations was also performed with Geneclass2. 92.9% of individuals sampled at MIH were referred to their own sample site. About 48.4% (N = 15) of the sampled individuals at SAN were assigned to their sample site, but 41.9% (N = 13) individuals were allocated to the founder population at MIH and 9.7% (N = 3) individuals to STM. The individuals of STM were assigned to their sample site with a percentage of 90.6% (N = 29). Over all populations, there was a quality index of 67.1% and a correct assignment rate of 78%. The test for first generation migrants revealed one potential migrant from SAN to STM.

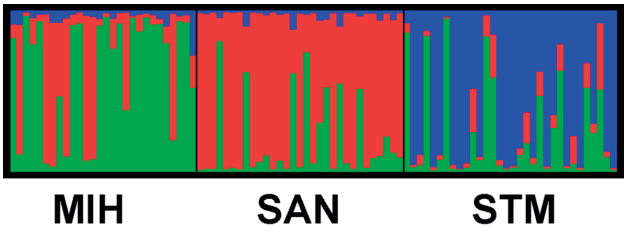

Figure 3. Estimation of the number of *Hyla arborea* populations for K = 3 using the program Structure ver. 2.3.1 (Pritchard et al. 2000). Each individual is represented by a single vertical line.

Genetic bottleneck

The Wilcoxon signed-rank test revealed a significant heterozygote excess as an indication for a recent bottleneck for the populations at MIH and SAN. The population at MIH also exhibited a shifted mode (Table 3), while there was no evidence for significant heterozygote excess, deficiency or a shifted mode at STM.

Discussion

Our results demonstrate how molecular analyses can give valuable information about the success of translocation studies. In the last years, genetic studies have been frequently used to evaluate the efficacy of translocation projects in conservational terms (reviewed in Germano & Bishop 2009). Thereby, microsatellites have proven to be a useful tool to genetically assess the newly formed populations consisting of translocated individuals. Although in many studies, translocated populations were found to suffer from bottlenecks and relatively low expected heterozygosities (Larson et al. 2002, Maudet et al. 2002), recent translocation studies on gopher tortoises (Tuberville et al. 2005), swift foxes (Moehrenschlager & Macdonald 2003), and the fire-bellied toad *Bombina bombina* (Schröder et al. 2012) have confirmed translocation to be

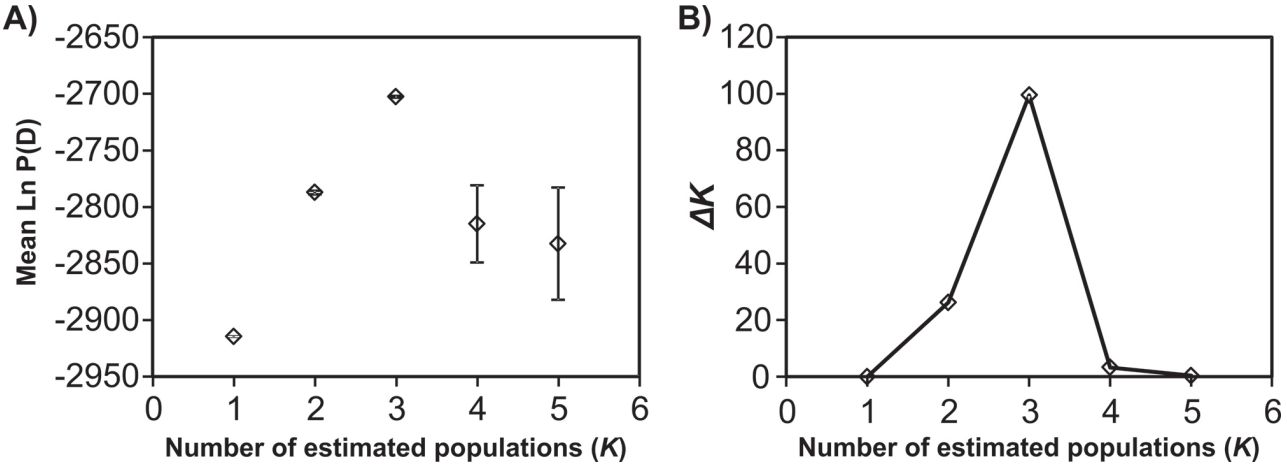

Figure 4. (A) Mean logarithm of probability of data [Ln P(D)] from K = 1 to K = 5 and (B) ΔK estimate with ΔK being the second order rate of change of the likelihood function with respect to K as recommended by Evanno et al. (2005) to detect real number of clusters, peak at K = 3.

Table 3. Results of bottleneck analysis. The table summarizes the p-value of one-tailed tests for heterozygote excess and the mode-shift. Null hypothesis: The population is at mutation-drift equilibrium. Significant values are in bolds (significance level: 0.05). MIH – Minderheide; SAN – Sachsenhäger Niederung; STM – Lake Steinhuder Meer.

Population	Wilcoxon signed-rank test for heterozygote excess			Mode-shift
	SSM in TPM = 95% variance = 12	SSM in TPM = 80% variance = 12	SSM in TPM = 70% variance = 30	
MIH	**0.003**	**0.003**	**0.001**	Shifted mode
SAN	**0.034**	**0.006**	**0.002**	Normal
STM	0.396	0.170	0.117	normal

an appropriate conservation method for endangered species. For these projects, the criteria for a successful translocation were moderate survival rates and litter sizes, as well as a relatively high genetic diversity assessed during subsequent monitoring.

Our study revealed that all three examined populations including the translocated one exhibited relatively high genetic diversities. Genetic diversity was examined as expected heterozygosity (H_e), allelic richness, and gene diversity. Our results for H_e are similar to those in other populations of *H. arborea* which were evaluated for the region of Hannover (Kirchhoff 2010: H_e = 0.57 to 0.74; Krug & Pröhl 2013: H_e = 0.58 to 0.77) and Switzerland (Angelone & Holderegger 2009: H_e = 0.27 to 0.71; Dubey et al. 2009: 0.49 to 0.66) in former surveys. Compared to studies on *H. arborea* in Denmark (Andersen et al. 2004 H_e = 0.35 to 0.53) and in the Netherlands (Arens et al. 2006 He = 0.39 to 0.59), expected heterozygosity and, therefore, genetic diversity determined in this study are at a higher level. In both Denmark and the Netherlands, the tree frog populations suffered from fragmentation and severe bottlenecks (Andersen et al. 2004, Arens et al. 2006) which might explain the low genetic diversity. In this study, the highest number of alleles and highest allelic richness were found at STM. This population is the result of a resettlement project of the ÖSSM, where tadpoles originating from different source populations in the region of Hannover in Lower Saxony and near Minden (MIH) in North-Rhine Westphalia were introduced (Brandt 2007, Taddey 2015). The genetic components of the different founder populations together with the large population size at this site probably contribute to the maintenance of high genetic diversity which is in line with the findings of other studies on *Hyla arborea* (Angelone & Holderegger 2009: gene diversity = 0.618 to 0.677; Broquet et al. 2010: gene diversity = 0.76 to 0.96). Interestingly, although tadpoles had been newly introduced at SAN in 2008 and the population might be affected by founder effects, the population exhibited only a small difference in genetic diversity compared to its source population. Therefore, we conclude that the number of introduced tadpoles was sufficient to maintain most of the original genetic diversity and that the habitat at SAN has provided optimal conditions for the establishment of an intact mirrored population. The number of calling males counted from 2008 to 2014 confirms that the population size is steadily increasing. However, there were signs of a recent genetic bottleneck in the mirrored (SAN) and its source population (MIH). In 2000, a strong reduction of the population at MIH was detected but single individuals persisted in a private pond. After the creation of several new breeding ponds, the remaining frogs resettled and expanded in MIH (Diesing 2015, personal correspondence), thus providing another example how management activities can help to revitalize depleted populations. Estimations of more than 100 calling males in more than ten ponds during the sampling sessions (Oswald & Taddey, personal observation) and the high genetic diversity examined in this study suggest that the population has recovered from breakdown in 2000 and is suitable for use as a donor population.

Analyses on genetic differentiation revealed a global F_{ST} of 0.08 which corresponds to the values obtained by Kirchhoff (2010, global F_{ST} = 0.121), Taddey (2015, global F_{ST} = 0.118) and Krug & Pröhl (2013, global F_{ST} = 0.106). In comparison to surveys on the European tree frog in the Netherlands (Arens et al. 2006, global F_{ST} = 0.18) and in Denmark (Andersen et al. 2004, global F_{ST} = 0.225), the values obtained for the region of Hannover were lower, indicating less differentiation. The discrepancy in global F_{ST} values between the different regions might be due to different sampling methods: In Hannover adult frogs were sampled from different ponds, whereas in Denmark and the Netherlands samples were taken from tadpoles (in case of Andersen et al. 2004 more than one tadpole per clutch). However, the low global F_{ST} in our study is in line with the fact that a small part of the genetic variation is located among populations. In contrast, pairwise F_{ST} and D_{est} measures were relatively high compared to other *Hyla* studies (Angelone & Holderegger 2009: F_{ST} = 0.033 to 0.099; Dubey et al. 2009: F_{ST} = 0.01 to 0.07) and exhibited significant differentiation between all of the three geographic populations. Therefore, each sample site is considered a distinct genetic population itself which coincides with the most likely population structure proposed by Structure (K = 3). The F_{ST} and D_{est} values between MIH and STM as well as between SAN and STM were higher than those between MIH and SAN. Likewise, Structure found admixture between the latter two sites. Similar results for differentiation indices between MIH and STM (F_{ST} = 0.073, D_{est} = 0.175) were found by Taddey (2015) who detected differentiation between all surveyed populations with some admixture between STM and its sever-

al source populations. The differentiation was considered as a result of admixture between all resettled frogs, therefore creating a genetic mosaic at STM that differs from its source populations. Since in our study the mirrored population (SAN) stem from one founder population (MIH) and was resettled in an area with no other populations, there was no possibility of migration or extensive genetic exchange with other frogs from the area. The rapid indication of divergence after only 10 years, however, corresponds to the findings of a study on microsatellites in *Bufo calamita* (Rowe et al. 1998) who detected genetic differentiation between founder and translocated populations after 10–15 years. They suggested that the genetic differentiation stems from bottleneck events or selection at linked loci. However, for this study the cause of differentiation remains unknown due to the lack of prior genetic analyses. The differentiation might result from changes in the mirrored population (SAN), but it might also be due to non-random sampling of the tadpoles from the source population at MIH that were used for translocation to SAN. Since the tadpoles stem from three nearby ponds, they are unlikely to represent the whole gene pool of MIH which has more than 10 ponds distributed in an area of 31 ha. For future studies, we recommend random sampling of several eggs or tadpoles from all viable ponds in the area. When investigating the microsatellite allelic pattern, the results are in concordance with the F_{ST} and D_{est} values. The number of private alleles supports the low differentiation between MIH and SAN which exhibited a similar allele composition. STM exhibited a rather unique allelic pattern corresponding to the higher degree of differentiation between this population and MIH or SAN. The higher genetic distance between MIH or SAN and STM might be due to the admixed genetic structure of STM which is a genetic mosaic of its different founder populations. In 2008, tadpoles of MIH have been introduced into ponds at SAN. Thus, SAN and MIH share a common genetic origin. Even though the geographic distance between SAN and STM is low (about 10 kilometres), the genetic differentiation contradicts the presence of extensive genetic exchange through migration between the two sample sites. Only one individual was considered as a first generation migrant from SAN towards STM. Further efforts should be undertaken to better connect SAN to the genetically diverse population at STM. Two single observations of tree frogs in private ponds in Wiedenbrügge which is located between the latter two populations (Brandt 2015, personal observation), indicate that tree frogs are dispersing in this area. The creation of further stepping stone habitats between Wiedenbrügge and SAN or STM could improve connectivity and establish migration. Constant migration would then counteract genetic depletion at SAN and improve the genetic situation and, therefore, survival and adaptability in the long term.

Translocations are still controversial in conservation biology, since, especially for amphibians and reptiles, success rates have been found to be very low and translocations were suggested to be unsuitable for these taxons (Dodd & Seigel 1991). However, the success rate has doubled within the last 15 years (Germano & Bishop 2009), indicating a positive trend to more successful translocations. Further research on this topic might give a better understanding of the effects of translocations on amphibian populations. We recommend conservation projects to be accompanied by regular genetic analyses, as they facilitate the choice of suitable founder populations, allow comparison of unbiased samples taken before and after translocation and enable immediate intervention in case of possible failures such as genetic depletion and bottleneck events. Translocation success is influenced by many factors such as stress (e.g., Mathies et al. 2001, Alberts 2007, Teixeira 2007), infections like the chytrid fungus (Fellers et al. 2007, Fisher & Garner 2007) which can lead to a depletion of the immune system, and ecological conditions at the release site (Griffith et al. 1989, Dodd & Seigel 1991). Thus, we strongly recommend periodic genetic and ecological monitoring of translocated as well as founder populations prior to, during and after translocations in order to maintain stable and growing populations.

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Appendix 1

Microsatellites used for genetic analyses. The first four loci with the prefix Ha- were isolated by Berset-Brändli et al. (2008). The other eight loci with the prefix WHA-1- were isolated by Arens et al. (2000).* annealing temperature; ** type of program: B (thermal profiles as recommended by Berset-Brändli et al. (2008)), NP (normal program), LP (long program). NP and LP were applied as recommended by Arens et al. (2000). PCR-conditions shown in bolds differ from the recommendations (parenthesized) of Arens et al. (2000). MIH = Minderheide, SAN = Sachsenhäger Niederung, STM = Lake Steinhuder Meer.

Locus	Repeat motif	Observed size range (bp)	Dye	PCR-conditions	Number of alleles		
					MIH	SAN	STM
Ha-A130	$(CA)_{10}...(CA)_{13}$	92–148	FAM	56.8*B**	2	2	3
Ha-B12	$(TC)_{21}$	236–256	HEX	56.8B	1	1	2
Ha-B5R3	$(TC)_{13}$	89–97	FAM	56.8B	4	4	7
Ha-D115	$(TAGA)_{16}$	194–214	FAM	56.8B	5	4	5
WHA1-9§	$(CA)_{20}$	99–141	FAM	60NP	6	5	6
WHA1-20§	$(GT)_{18}$	184–194	NED	64.6NP (55NP)	6	4	5
WHA1-25§	$(GT)_{20}$	101–113	FAM	59.5NP (55NP)	6	3	5
WHA1-60	$(GT)_{22}$	153–173	NED	55LP	7	5	7
WHA1-67§	$(CA)_{21}$	194–250	HEX	59LP (55LP)	4	5	9
WHA1-103§	$(GT)_{21}$	235–249	HEX	60NP	4	3	5
WHA1-104	$(GT)_{22}$	263–293	HEX	60NP	7	5	6
WHA1-140§	$(GT)_{25}$	109–203	FAM	55NP	6	5	7

Appendix 2

PCR programs with different time periods and number of cycles which include step two (94°C) to four (72°C). The DNA denaturises at a temperature of 94°C. The annealing occurs at the primer-specific annealing temperature T_a. The elongation is performed at a temperature of 72°C. NP and LP were applied as described by Arens et al. (2000). The program B follows recommendations by Berset-Brändli et al. (2008).

Program	Temperature (°C)	Time period	Cycles
NP: normal program	94	3 min	
	94	15 sec	
	T_a	45 sec	35
	72	60 sec	
	72	20 min	
LP: long program	94	3 min	
	94	45 sec	
	T_a	45 sec	35
	72	105 sec	
	72	20 min	
B: thermal profiles according to Berset-Brändli et al. (2008)	94	5 min	
	94	45 sec	
	T_a	45 sec	35
	72	60 sec	
	72	5 min	

Appendix 3

Information on private alleles per locus and per population. Npa = number of private alleles, Fpa = frequency of private alleles. The frequency for more than one private allele per locus was averaged over number of private alleles at the respective locus. MIH = Minderheide, SAN = Sachsenhäger Niederung, STM = Lake Steinhuder Meer.

Locus	MIH		SAN		STM	
	Npa	Fpa	Npa	Fpa	Npa	Fpa
Ha-A130	0	0	0	0	1	0.016
Ha-B12	0	0	0	0	1	0.017
Ha-B5R3	0	0	0	0	3	0.026
Ha-D115	0	0	0	0	1	0.031
WHA1-9§	0	0	0	0	0	0
WHA1-20§	1	0.018	0	0	0	0
WHA1-25§	1	0.018	0	0	0	0
WHA1-60	0	0	0	0	0	0
WHA1-67§	0	0	0	0	4	0.043
WHA1-103§	0	0	0	0	1	0.048
WHA1-104	1	0.018	0	0	0	0
WHA1-140§	1	0.018	1	0.097	2	0.1175