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Population genetics suggests effectiveness of habitat connectivity measures for the European tree frog in Switzerland

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Summary

1. Governmental authorities in many countries financially support the implementation of habitat connectivity measures to enhance the exchange of individuals among fragmented populations. The evaluation of the effectiveness of such measures is crucial for future management directions and can be accomplished by using genetic methods.

2. We retraced the population history of the European tree frog in two Swiss river valleys (Reuss and Thur), performed comprehensive population sampling to infer the genetic structure at 11 microsatellite markers, and used first-generation migrant assignment tests to evaluate the contemporary exchange of individuals.

3. Compared with the Thur valley, the Reuss valley has lost almost double the number of breeding sites and exhibited a more pronounced genetic grouping. However, similar numbers of contemporary migrants were detected in both valleys. In the Reuss valley, 81% of the migration events occurred within the identified genetic groups, whereas in the Thur valley migration patterns were diffuse.

4. Our results show that the connectivity measures implemented in the Reuss valley facilitated effective tree frog migration among breeding sites within distances up to 4 km. Nevertheless, the Reuss valley exhibited high genetic differentiation, which reflected the impact of barriers to tree frog movement such as the River Reuss. By contrast in the Thur valley, a larger number of breeding sites have been preserved and high admixture indicated exchange of individuals at distances up to 16 km.

5. *Synthesis and applications*. We show that genetic methods can substantiate the effectiveness of connectivity measures taken in conservation management at the landscape scale. We urge responsible authorities from both river valleys to continue implementing connectivity measures and to create a dense network of breeding sites, as spatial gaps of 8 km are rarely traversed by tree frogs.

Key-words: conservation, dispersal, first-generation migrant, fragmentation, genetic structure, genotype assignment, *Hyla arborea*, microsatellites, stepping-stone

Introduction

The continuous modification of landscapes by human activities leads to the damage and loss of natural habitats as well as to their fragmentation. Although conservation areas have been safeguarded in many countries, they often are spatially isolated remnants in otherwise intensively used landscapes. The effective isolation of such remnant habitat patches results from barriers to movement for inhabiting species and represents a particular challenge to nature conservation (Lindenmeyer & Fisher 2006). Roads, for instance, cause high mortality due to collision with vehicles (Trombulak & Frissell 2000) and such barriers not only interrupt migration, they also prevent the colonization of suitable but unoccupied habitat patches (Bowne & Bowers 2004). Habitat fragmentation may furthermore lead to increased genetic subdivision of populations, higher inbreeding and the loss of genetic diversity within populations (Allendorf & Luikart 2007; Keyghobadi 2007). Several types of measures to increase the connectivity among remnant habitats and populations are implemented by conservation managers such as protected greenways and ecological buffer zones like hedgerows or extensively used agricultural grasslands (Jongman & Pungetti 2004).

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In the Swiss lowlands, urban sprawl, intensive agriculture and a dense traffic infrastructure are causing extreme habitat fragmentation (Jaeger *et al.* 2008). To counteract this development, Swiss federal and regional authorities financially support the implementation of habitat connectivity measures. Their aim is to enhance or re-establish the exchange of individuals among populations in fragmented landscapes. The first priority thereby is to save existing habitat patches, and secure or increase their quality (i.e. the nodes of a network; Moilanen *et al.* 2005), and secondly, to set up dispersal corridors or stepping-stones between existing patches (i.e. the meshes of a network; Bennet 1999). Several species-specific projects have been initiated and aimed at establishing functional connectivity among populations, including projects on the European tree frog *Hyla arborea* L. in Eastern Switzerland.

One goal of applied ecology is to evaluate the effectiveness of connectivity measures (Beier & Noss 1998). The underlying question is whether structural connectivity measures also provide functional connectivity, whereby there is effective exchange of individuals (and thereby genes) among populations (Baguette & Van Dyck 2007). However, the assessment of functional connectivity is methodologically difficult, because direct observations or mark–recapture surveys of migration are time- and labour-intensive (Bowne & Bowers 2004). Evaluations of effectiveness thus mostly rely on monitoring trends in population size (Joseph *et al.* 2006) or recording the use of connectivity elements such as underpasses or wildlife crossings (Haddad *et al.* 2003). One drawback of these methods is that they do not evaluate the effective exchange of individuals among populations leading to gene flow (Horskins, Mather & Wilson 2006; Strasburg 2006). In the case of the tree frog, it is unclear whether the connectivity measures taken in Eastern Switzerland provide functional connectivity at the landscape level, despite the documentation of substantial movement distances in the species (Arens *et al.* 2006) and its colonization of newly created ponds (Rieder-Schmid 2002; Tester & Flory 2004). However, contemporary or recent migration and gene flow can be studied using population genetic approaches such as assignment tests based on individual multilocus genotypes (Manel, Gaggiotti & Waples 2005). Assignment methods allow the detection of contemporary migration events by classifying individuals as migrants as well as identifying their most likely population of origin (Paetkau *et al.* 2004; Piry *et al.* 2004). When sampling all or the majority of the populations within a landscape, this approach represents a powerful tool to evaluate the success of connectivity measures taken in conservation management (Berry, Tocher & Sarre 2004; Manel *et al.* 2005).

Our goal was to evaluate the effectiveness of structural connectivity measures (i.e. the implementation of stepping-stone habitats) for the European tree frog in two Swiss landscapes, the Reuss and Thur valley, separated by 150 km and differing in the level of former population decline and conservation measures implemented. We retraced the population histories, inferred the genetic structure and used assignment tests to evaluate contemporary migration among populations in comprehensive samples of the two landscapes studied. Our hypothesis was that in the Reuss valley, where recent population decline was serious but many stepping-stone habitats had been established, we should find contemporary migration among populations and a genetic clustering reflecting a recent expansion out of several source areas. In contrast, in the Thur valley, where population decline was less severe and only few connectivity measures had been implemented, we expected to encounter a genetically homogenous metapopulation structure due to historical and contemporary migration.

Materials and methods

STUDY SPECIES AND LANDSCAPES

The European tree frog is a pioneer species that was once widespread in the Swiss lowlands before it declined to less than half of its former distribution area in the 1980s (Zumbach 2004). The decline was caused by massive habitat destruction and was additionally strengthened by the closure and infilling of gravel-pits, which are secondary breeding habitats for tree frogs. Since the 1980s, the decline has continued due to dense settlements and roads having a negative effect on tree frog presence (Pellet, Guisan & Perrin 2004). The European tree frog is consequently listed as an endangered species in Switzerland (Schmidt & Zumbach 2005).

In the Reuss river valley in Eastern Switzerland, the tree frog has experienced such a devastating population decline that a specific conservation project was launched by Weidmann & Flory (1991). Since then the remaining breeding habitats have been protected and managed according to tree frog requirements (i.e. preserving pioneer conditions). Stepping-stone habitats have been established to provide migration routes between existing breeding sites and to increase overall population size (Tester & Flory 2004). Since 1994, all habitats occupied by tree frogs have been monitored annually, with each breeding site visited three times during the breeding season to estimate the size of male choruses. Total chorus size in the Reuss valley has increased from *c.* 500 calling males in 1994 to *c.* 1100 calling males in 2006 (C. Flory, unpublished data; C. Bühler, unpublished data).

In the Thur valley, the river Thur has been secured with dams resulting in the riparian area largely becoming unsuitable as a tree frog habitat. In the 1980s, many breeding sites to the north and south of the river were protected (Beerli 1985) leading to increased tree frog population sizes (Cigler, Lippuner & Meier 2002; Rieder-Schmid 2002). Today, the Thur valley forms part of Switzerland's largest continuous area inhabited by the tree frog (Zumbach 2004). However, no monitoring programme has been put in place, and connectivity measures to increase tree frog migration were only implemented from 1999 onwards (Cigler *et al.* 2002; Rieder-Schmid 2002). Compared with the Reuss valley, connectivity measures were taken later, but tree frog habitats have been protected earlier.

POPULATION HISTORY

To assess the population history of *H. arborea* in the two study landscapes, we chose three different time periods. For the Reuss valley, we took information from: (1a) two inventories from 1991 and 1993 (Cigler 1993; Flory 1999), (2a) chorus size data from 1999 (C. Flory, unpublished data; C. Bühler, Hintermann & Weber, unpublished data) and (3a) chorus size data from 2006 (C. Flory, unpublished data; C. Bühler, unpublished data) combined with our own sampling data. For the Thur valley, we selected data from (1b) an inventory of 1994 (Kaden & Meienberger 1995), (2b) two inventories carried out in 1998 and 2002 (Cigler *et al.* 2002; Rieder-Schmid

2002) and (3b) our own sampling data from 2007. When calling males were present in (1a/1b), (2a/2b) and (3a/3b), a breeding site was considered as an old one. When calling males were present in (2a/2b) and/or (3a/3b), the breeding site was considered as newly colonized. When calling males were absent in (2a/2b) and (3a/3b) or (3a/3b) only, the corresponding breeding site was considered extinct. Chorus size data from 2006 and 2007 were used to assign breeding sites to two classes with < 30 or > 30 calling males respectively.

SAMPLE COLLECTION AND DNA EXTRACTION

We sampled 34 of the 36 tree frog breeding sites in the Reuss valley in 2006 and 29 of the 47 sites in the Thur valley in 2007 (Fig. 1). When no males were calling at sites where tree frogs had formerly been reported, we visited the site three times before assuming the frog to be extinct at that site. Otherwise, we determined the chorus size per site, which generally agreed with numbers from recent monitoring data. We caught as many individuals as possible at sites with less than 30 calling males, and 30 individuals at sites with more than 30 calling males. Occasionally, we were able to catch females, which are distinguishable from males by the absence of vocal sacs. We took non-invasive buccal swabs from each frog for genetic analysis (Copan Italia S.p.A., Brescia, Italy; Broquet *et al.* 2007) and photographs from both lateral sidelines. The green back and the light-coloured belly of *H. arborea* are separated by a dark lateral stripe, which allows the identification of individuals. Buccal swabs were stored at −20 °C until DNA extraction using the DNeasy Tissue Kit (QIAGEN, Hilden, Germany) following the protocol of Broquet *et al.* (2007). DNA was eluted twice with 100 μL of AE buffer (QIAGEN, Hilden, Germany).

MICROSATELLITE ANALYSIS

Ten individuals from different sites were screened for polymorphism using nine microsatellite loci from Arens *et al.* (2000) and eight loci from Berset-Brändli *et al.* (2008). Eleven primers were finally selected owing to clear patterns and consistent amplification and polymorphism in both study regions. Microsatellites were amplified using fluorescently labelled primers in four multiplex polymerase chain reactions (PCR), which were performed in 7-μL reaction volumes containing 3 μL of template DNA (10–40 ng μL^{-1}), 1× Multiplex PCR Master Mix (QIAGEN) and 0·2–0·7 μM of each forward and reverse primer. Multiplex 1 consisted of primers WHA1-9 and WHA 1-103 (both 0·7 μM); multiplex 2 of primers WHA 1–104 and WHA 1–140 (both 0·7 μM); multiplex 3 of primers WHA 1–20 (0·3 μM), WHA 1–25 (0·4 μM) and WHA 1–67 (0·6 μM); and multiplex 4 of primers Ha-A127 (0·4 μM), Ha-D115 (0·6 μM), Ha-B5R3 and Ha-E2 (both 0·3 μM). Multiplex PCRs were carried out on PTC-100 Thermocyclers (MJ Research, Waltham, Massachusetts, USA) with polymerase activation at 95 °C for 15 min, followed by 30 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C (multiplexes 2 and 3), 58 °C (multiplex 4) or 60 °C (multiplex 1) for 90 s and extension at 72 °C for 90 s, ending with a final extension at 72 °C for 10 min. Amplification products were run against 500 ROX™ size standard on an ABI 3130 automated sequencer (Applied Biosystems, Carlsbad, California, USA), and resulting peaks were visualized and scored using GENEMAPPER 3.7 (Applied Biosystems, Carlsbad, California, USA).

DATA ANALYSIS

Multilocus genotypes were screened for repeated occurrences using the program GENALEX 6 (Peakall & Smouse 2006). Samples with matching genotypes were evaluated for repeated capture of the same individual by checking photographs of lateral stripes. In subsequent analyses, only genotype data from different individuals were used.

We tested all pairs of loci across sites within river valleys for linkage disequilibrium using the log-likelihood statistic G implemented in FSTAT 2.93 (Goudet 2001) and applying sequential Bonferroni correction (Rice 1989). Conformity to Hardy–Weinberg equilibrium was assessed with exact U-tests implemented in GENEPOP 4.0 (Raymond & Rousset 1995), which uses a Markov chain method to estimate significance.

As measures of genetic diversity, we calculated the mean number of alleles (A), observed heterozygosity (H_o) and expected heterozygosity (H_e) per site in GENETIX 4.03 (Belkhir *et al.* 1996). We used two general linear models in SPSS 10.0.1 (SPSS 2001) to test for differences in H_o and H_e between the Reuss and the Thur valley (fixed factor), with either current chorus size or A (as a measure of long-term population size; Allendorf & Luikart 2007) as covariates. The Spearman rank correlations between the two covariates and between the two dependent variables were determined. Since these correlations were not very high ($r_s \leq 0{\cdot}644$), we retained all parameters in the models.

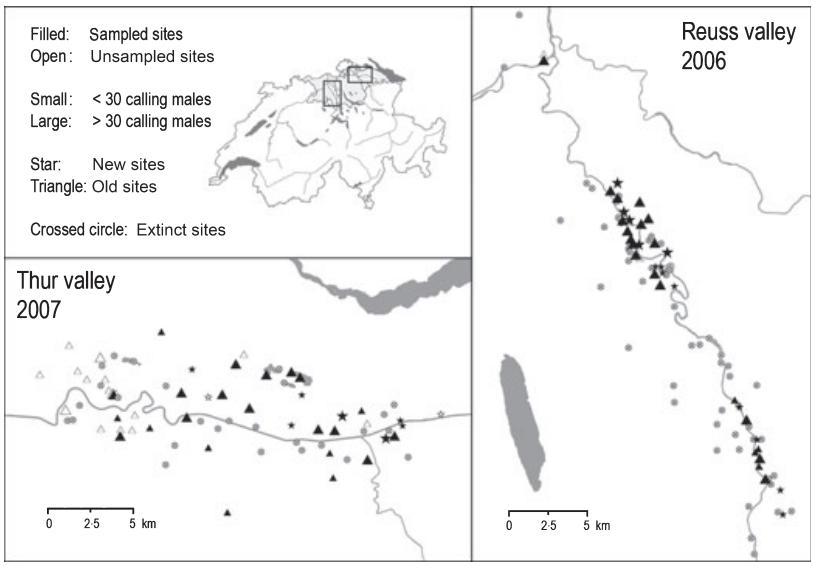

Fig. 1. Summary of *Hyla arborea* population history within the Reuss and Thur valleys in Switzerland. Old sites denote breeding sites documented in 1991/1993 and persisting until 2006/2007. New sites denote sites originating after 1991/1993 and extinct sites are sites no longer documented after 1991/1993. Sampled sites were genetically analysed.

To estimate the effect of spatial isolation on the genetic structure of *H. arborea*, we performed isolation-by-distance tests between genetic differentiation among sites ($F_{ST}/1 - F_{ST}$) and log-transformed geographical distances within each river valley. Mantel tests were calculated with 1000 permutations in ARLEQUIN 3.1 (Excoffier, Laval & Schneider 2005). We then calculated overall F_{ST}-values and their standard errors by jackknifing over loci in FSTAT 2.93 (Goudet 2001) and inferred spatial genetic structure using STRUCTURE 2.2 (Pritchard, Stephens & Donnelly 2000). For the latter, we performed 10 independent runs per predefined cluster number ($K = 1$–15) using the admixture model without prior population information at burn-in lengths of 100 000 and 150 000 Markov-chain Monte Carlo sampling repeats. We determined K following the STRUCTURE manual guidelines (Pritchard *et al.* 2000).

In a separate analysis, we performed first-generation migrant tests in GENECLASS2 (Piry *et al.* 2004), to estimate contemporary migration events in the two river valleys. This test identifies migrants as individuals that were born at a breeding site other than the one in which they were sampled. Since we had sampled the majority of potential source sites, we used the ratio $L = L_{home}/L_{max}$ as the statistical criterion for the likelihood computation (L_{home} being the likelihood computed for the site where an individual was sampled and L_{max} being the highest likelihood value among all available sites including the site where the individual was sampled; Paetkau *et al.* 2004). We used the partially Bayesian method of Rannala & Mountain (1997) in combination with the Monte Carlo resampling algorithm of Paetkau *et al.* (2004) to determine the critical value of the test statistic at $\alpha = 0{\cdot}01$. Finally, we calculated rough estimates of contemporary migration rates for each valley by dividing the number of individuals identified as migrants by the respective sample size.

Results

We evaluated the population history of 92 breeding sites in the Reuss valley and 74 breeding sites in the Thur valley (Fig. 1). In the Reuss valley, 25% were old (23 sites; 21 sampled), 14% were new (13 sites; all sampled) and 61% of the sites were extinct (totally 56). All new sites had been created between 1993 and 2005 to act as stepping-stone habitats. They were mostly colonized by tree frogs 1 year after construction, with the exception of sites R33 and R34 (Table S1), where tree frogs from the same region were introduced in 2000. In the Thur valley, 51% of the sites were old (38 sites; 22 sampled), 12% were new (9 sites; 7 sampled) and only 37% were extinct (27 sites). Of the new sites, T4 and T15 were discovered in 2002 and T11, T12 and T17 in 2007 (Table S1). Only sites T26 and T28 were created in 2000 and 2004 respectively to act as stepping-stones. They were colonized by tree frogs during the following breeding season. Current chorus sizes with more than 30 calling males were identified in 47% and 55% of the sites in the Reuss and Thur valley respectively.

The buccal swabbing method was efficient, as all but one sample successfully amplified in PCR. Only two pairs of samples had matching multilocus genotypes, and the comparison of lateral stripes revealed that these samples stemmed from two individuals recaptured at different breeding sites in the Reuss valley (current migration). One individual moved 0·75 km (straight-line distance) from sites R10 to R11, and the other moved 1 km from sites R16 to R17. Total sampling size therefore consisted of 1169 individuals (completely genotyped at 11 loci), of which 34 were females. There was no significant linkage disequilibrium at any locus, and only sites R21 and R22 of the Reuss valley expressed significant deviations from Hardy–Weinberg equilibrium (Table S1).

In both river valleys, we found high levels of neutral genetic diversity: the mean numbers of alleles (A) ranged from 1·55 to 7·64, expected heterozygosities (H_e) from 0·27 to 0·71 and observed heterozygosities (H_o) from 0·45 to 0·77 per site (Table S1). Global gene diversity was lower in the Reuss than in the Thur valley ($H_e = 0{\cdot}618 \pm 0{\cdot}037$ SE vs. $0{\cdot}677 \pm 0{\cdot}053$ SE). In the general linear models, log chorus size showed a significant positive relationship with H_e ($F_{1,59} = 147{\cdot}626$, $P \leq 0{\cdot}001$) and H_o ($F_{1,59} = 4{\cdot}933$, $P = 0{\cdot}030$), hence revealing a clear dependence of neutral genetic diversity from chorus size (Fig. 2). For H_e, both the effects of region and the interaction between region and log chorus size were significant ($F_{1,59} = 18{\cdot}448$, $P \leq 0{\cdot}001$ and $F_{1,59} = 5{\cdot}357$, $P = 0{\cdot}024$ respectively). For H_o, there was neither a significant effect of region nor of the interaction. The general linear models using

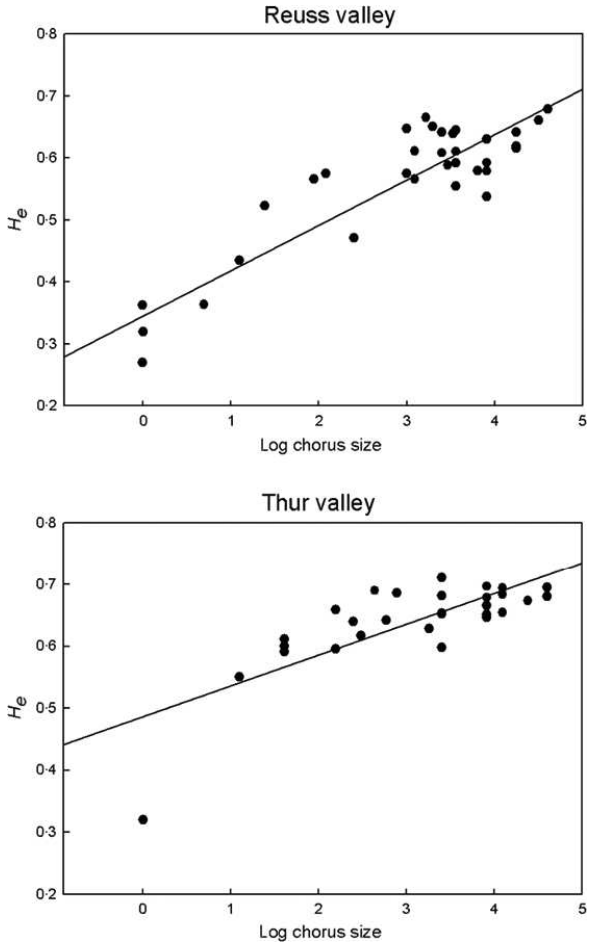

Fig. 2. Scatterplots of log chorus size and expected heterozygosity (H_e) in 34 *Hyla arborea* breeding sites from the Reuss and 29 sites from the Thur valley in Switzerland.

allele diversity as a covariate resulted in qualitatively identical results (data not shown).

Overall genetic differentiation was higher in the Reuss (F_{ST} = 0·099 ± 0·008 SE) than in the Thur valley (F_{ST} = 0·033 ± 0·004 SE), although the geographic distances among sites in the Reuss valley were generally smaller (Fig. 1). Significant isolation by distance was found in both river valleys, but it was more pronounced in the Reuss (r_m = 0·572, P < 0·001) than the Thur valley (r_m = 0·293, P = 0·017).

In the Reuss valley, the values of the mean logarithm of probability of the data [Ln $P(X \mid K)$] from STRUCTURE runs reached a plateau at K = 6, after which the spatial genetic clustering stabilized and the standard deviations per K started to increase (Figs S1 and S2). We therefore chose K = 6 as the number of clusters best capturing the geographical clustering of the breeding sites in the Reuss valley (Fig. 3). The proportion of membership (q-mean) of sites to belong to either of these six clusters was 0·95 in cluster 1 (1 site), ranged from 0·32 to 0·78 in cluster 2 (8 sites), 0·44 to 0·81 in cluster 3 (6 sites), 0·53 to 0·85 in cluster 4 (5 sites), 0·79 to 0·97 in cluster 5 (5 sites) and from 0·87 to 0·97 in cluster 6 (9 sites). Clusters 2 and 3 showed the highest admixture since q-mean values were below 0·5 for some sites. In contrast, proportions of membership were high for clusters 1 and 6 (above 0·8), which were separated by *c*. 8 km linear distance from the populations in the centre of the Reuss valley. A substantial fraction of the two introduced sites R33 and R34 (0·85 and 0·77 respectively), located southernmost of the Reuss valley, matched with cluster 4 (Fig. 3).

In the Thur valley, a maximum of mean Ln $P(X \mid K)$ was found at K = 3 and the geographical clustering pattern remained unchanged for all runs with K > 3 (Figs S1 and S3). We therefore chose K = 3 as the number of clusters best describing the genetic grouping in the Thur valley (Fig. 3). The q-mean values of these three clusters ranged from 0·18 to 0·64 in cluster 1 (24 sites), 0·74 to 0·84 in cluster 2 (4 sites) and was 0·83 in cluster 3 (1 site). Cluster 1 covered almost the whole area of the sampled region and showed high admixture, since 20 of the 24 sites had a q-mean value below 0·5. In contrast, the proportions of memberships of clusters 2 and 3 were much higher (note that at site T20 a single individual was genotyped). Cluster 2 was located south of the river Thur in the easternmost part of the valley. Cluster 3 consisted of site T29 only, situated southernmost of the valley and separated by *c*. 4–6 km from neighbouring sites.

The first-generation migrant tests detected 26 migrants across the Reuss valley and 24 in the Thur valley. This resulted in migration estimates of 4·5% and 4·1% respectively. Unsurprisingly, most of the contemporary migration events in the Reuss valley (81%) occurred among breeding sites within the genetic clusters identified by STRUCTURE (Figs 3 and 4) at linear distances ranging from 0·3 to 4·0 km. Of these events, 54% occurred among old sites, 42% among old and new sites, and

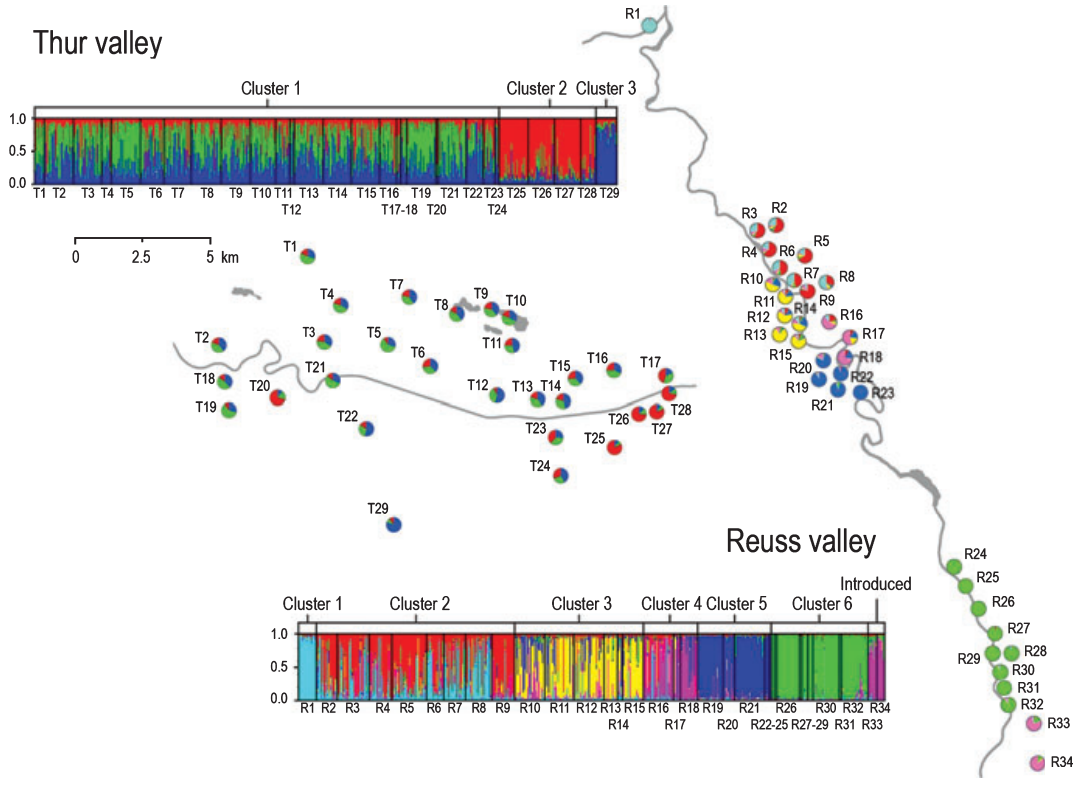

Fig. 3. STRUCTURE clusters of *Hyla arborea* in the Reuss (R1–R34) and Thur (T1–T29) valley in Switzerland. The colours within bars show the proportion of membership of each individual to the genetic clusters for each valley separately. The pie charts give the genetic membership per breeding site. For site abbreviations see Table S1.

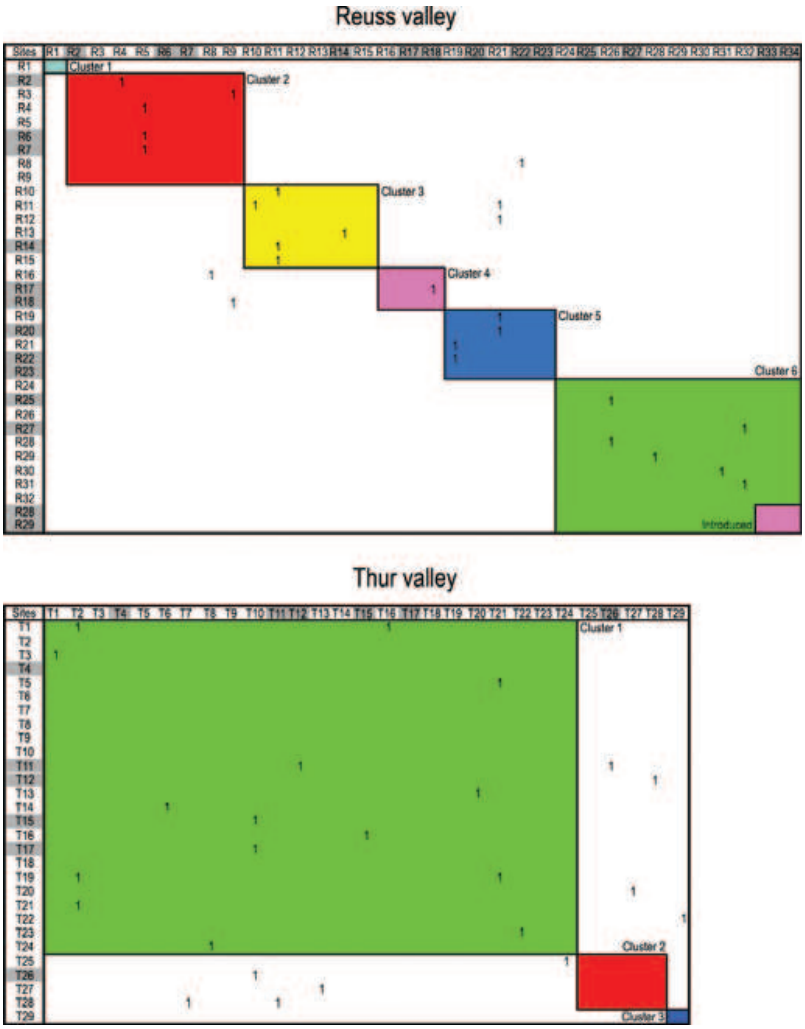

Fig. 4. Migration among *Hyla arborea* breeding sites detected by first-generation migrant tests. Sites listed in columns are the immigrant sites and those in rows are the source sites. Highlighted in grey are new sites whereas the others are old. The coloured frames highlight the STRUCTURE clusters as given in Fig. 3. For site abbreviations see Table S1.

only 4% among new sites (Fig. 4). Nevertheless, five migration events occurred between different clusters: four among clusters on the same side of the river Reuss at linear distances of 1·6–4 km, and one among site R8 from cluster 2 and site R22 from cluster 5 being separated by the river Reuss at a linear distance of 3·6 km. Two of the five migration events among clusters involved new sites. There was no migration between the spatially isolated clusters 1 and 6 with any of the sites in the centre of the Reuss valley (Fig. 4). There were also no migration events between the introduced southernmost sites R33 and R34 and other sites in cluster 6.

In the Thur valley, most of the contemporary migration events (62·5%) occurred among breeding sites within the highly admixed cluster 1. The migration events between sites occurred at linear distances ranging from 1·5 to 16 km, also across the river Thur (Fig. 4). Sixty-two per cent of migration events happened between old sites, 21% between old and new sites, and 17% between new sites. There were no migration events detected between the breeding sites within genetic cluster 2, having a size of only 3 km. Nine migration events occurred between clusters: eight between sites of clusters 1 and 2 at linear distances ranging from 2·25 to 16 km (also crossing the river Thur), and one between site T22 of cluster 1 and site T29 of cluster 3, two neighbouring sites south of river Thur at a linear distance of 3·7 km. Four of the contemporary migration events between clusters involved new sites.

Discussion

We found strong differences in the level of genetic diversity and differentiation of tree frog breeding sites between the two Swiss river valleys of Reuss and Thur, although the geographical scales sampled were similar. The level of genetic variation in *H. arborea* highly depended on male chorus size, and this relationship was much more pronounced in the Reuss than the Thur valley (Fig. 2). In both landscapes, only two sites harboured more than 100 calling males (Table S1). Thus, the loss of genetic variation in small populations could generally be interpreted as a consequence of genetic drift and possibly inbreeding (Allendorf & Luikart 2007). This particularly held for the Reuss valley, where tree frogs had experienced a severe population decline. Until the early 1990s, the Reuss valley had lost 61% of its breeding sites and today harbours fewer old breeding sites (25%) than the Thur valley (51% old sites and

37% extinct), a fact that could also explain the lower overall gene diversity in the Reuss when compared with the Thur valley (H_e = 0·618 vs. 0·677). Furthermore, the genetic subdivision of the Reuss valley was threefold (F_{ST} = 0·099 vs. 0·033), and isolation by distance was nearly twice as pronounced as in the Thur valley (r_m = 0·572 vs. 0·293). In accordance, tree frog sites in the Reuss valley exhibited substantial genetic clustering, while in the Thur valley most sites showed high admixture (Fig. 3). The higher level of genetic differentiation observed in the Reuss valley may reflect the impact of prominent barriers to movement, such as the river Reuss itself or gaps in the spatial distribution of tree frog sites (Funk *et al*. 2005). However, the differentiation may also have been caused by founder events with subsequent local expansion (Newman & Squire 2001).

For a scenario of genetic drift or effects of founder events in the Reuss valley, theory predicts that if isolated populations lose genetic variation due to drift, genetic distance among them should increase quickly (Hedrick 1999). If in the Reuss valley, small groups of protected breeding sites of the tree frogs became reproductively separated from each other and later on, after having recovered from the decline of the 1980s, acted as recolonization sources for newly established surrounding stepping-stone sites, this expansion on a small spatial scale will have resulted in groups of source and sink populations that were genetically similar within but different among groups (Hanski & Gagiotti 2004). Two findings that support such a recent expansion in the Reuss valley are that breeding sites were assigned to six different genetic clusters on a small spatial scale (Fig. 3) and that gene flow occurred predominantly within these clusters among both old and new sites (Fig. 4). The latter finding was also supported by the two recaptured individuals that both moved between breeding sites located within the same cluster. Nevertheless, there was evidence for some contemporary genetic exchange among clusters, but only among clusters in the central part of the Reuss valley and primarily located on the same side of the river Reuss. Gibbs (1998) suggested that rivers act as barriers to amphibian movement. The river Reuss, 60 m wide and with a strong current, seems to represent an obstacle to tree frog migration and dispersal.

The contemporary migration events detected in the Reuss valley occurred over straight-line distances of 0·3–4 km, a range that is in accordance with migration distances found in other studies on *H. arborea* (Vos, Ter Braak & Nieuwenhuizen 2000; Arens *et al*. 2006) as well as reported for amphibians in general (Smith & Green 2005). As discussed above, no migration was detected between the central parts of the Reuss valley and the marginal clusters 1 and 6, indicating that a distance above 8 km is not exceeded by *H. arborea* on a regular basis, at least not in a landscape heavily disturbed by human activities. A special situation arose in the two southernmost sites R33 and R34, where tree frogs were introduced in 2000. The individuals of these two sites were assigned to cluster 4 in the central Reuss valley (Fig. 3). The most likely source sites for this introduction were sites R16 or R17, as site R18 was newly created and only colonized in 2001 (H. Cigler, unpublished data; C. Flory, unpublished data). Although we detected no contemporary migration event among sites R32, R33 and R34, STRUCTURE analysis showed one individual at site R32 to be genetically related with the gene pool of cluster 4 (57%; Fig. 3), a result pointing to former migration events.

The Thur valley revealed a different genetic scenario. Although we found nearly as many contemporary migrant events in the Thur as in the Reuss valley, they occurred over almost the entire area sampled at distances of 1·5–16 km (Figs 3 and 4). Moreover, many migration events happened between sites situated on opposite sides of the river Thur, which is a shallower river than the river Reuss and only has a width of *c*. 30 m. This would imply that tree frogs disperse over considerable distances across the landscape and are able to cross the river Thur on a regular basis. Such regular gene exchange was also reflected in the high genetic admixture of cluster 1 (Fig. 3). Although a maximum migration distance of 12·5 km has been reported for the European tree frog, such migration distances are believed to be exceptional, especially in fragmented landscapes (Pellet *et al*. 2004; Arens *et al*. 2006). A review covering 102 studies on 53 anuran species revealed that the majority (56%) of movement distances are below 1 km, while 44% range from 1 to 10 km, and only 7% involve distances larger than 10 km, with an average movement of 2·02 km (Smith & Green 2005). In the Thur valley, this average movement distance was already exceeded by the most isolated site T29, separated from others by 3·7–6·5 km, which had received a migrant from its nearest site T22. Given these large movement distances, it is surprising that no contemporary migration was detected among the sites within cluster 2, the size of which was similar to the clusters found in the Reuss valley. All the sites within cluster 2 were constructed or reshaped during 2000 and 2004 and subsequently colonized by tree frogs (J. Rieder-Schmid, unpublished data), leading to a similar founder effect as described in the Reuss valley. The most likely explanation for the lack of detected contemporary migration events in cluster 2 is the low power of assignment tests in cases where population differentiation (F_{ST}), sample sizes and the number of loci studied are low (Manel *et al*. 2005). According to Cornuet *et al*. (1999), accurate assignment can be achieved by using at least 10 microsatellite loci on 30 individuals per population with an F_{ST} value near 0·1. In our study, these conditions were fulfilled in the Reuss valley, but not necessarily in the Thur valley and certainly not in cluster 2 with a very low F_{ST} value of 0·019.

Our genetic analyses provide compelling evidence that the conservation and connectivity measures taken for the tree frog in the Reuss valley have been successful: population decline has been stopped, migration among breeding sites at distances of up to 4 km is warranted and tree frogs have expanded their range. However, the gene pools in the Reuss valley are not yet mixed, since migration among clusters is still weak and sometimes completely missing (Fig. 4). By contrast, in the Thur valley, a larger number of breeding sites have been preserved, and they retained a genetic structure indicative of gene flow still contributing to the mixture of historically well connected gene pools (Fig. 3). However, one should note that it is exactly the

genetic similarity among sites that causes low resolution power in assigning first-generation migrants. It would therefore be relevant to prove contemporary migration and functional connectivity in the Thur valley by either increasing the number of loci or carrying out alternative methods such as mark–recapture experiments, at least at smaller spatial scales.

We conclude that our genetic approach was successful in proving the effectiveness of connectivity measures taken in the conservation management of the European tree frog in eastern Switzerland. The genetic results not only confirm that newly established ponds are quickly colonized by tree frogs, they, moreover, suggest that these ponds are subsequently incorporated into a habitat network connected by considerable individual exchange. Establishing stepping-stone habitats is therefore a successful strategy that could be adopted for other pond-breeding organisms. In doing so, attention should be given to providing high-quality habitats for the target species within reachable distances and across permeable landscapes. In the case of the tree frog, distances >8 km are rarely traversed by single individuals, suggesting that effective habitat networks for the species must include closely spaced refuges at 1–2 km. We therefore urge the authorities responsible for both the Thur and Reuss valleys to continue implementing connectivity measures with the long-term prospect of connecting genetic clusters. As a next step, contemporary tree frog movement should be evaluated in a landscape genetic approach (Holderegger & Wagner 2008), focussing on those landscape elements potentially forming obstacles or barriers to dispersal.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Mean posterior probability plots.

Fig. S2. STRUCTURE analysis for the Reuss valley.

Fig. S3. STRUCTURE analysis for the Thur valley.

Table S1. Breeding site information and genetic diversity indices

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