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Conservation Genetic status of Moor Frog in France

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CONSERVATION GENETIC STATUS OF MOOR FROG (*RANA ARVALIS*) IN FRANCE

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Abstract

The western range edge of the distribution of the moor frog, *Rana arvalis*, is situated in northern France and Belgium. With just four poorly known remaining populations in France this species is on the brink of extinction in France. In Belgium, the species has a slightly broader distribution in the NE sandy region of Campines, occurring in circa 30 sites, of which only a handful have a favourable conservation status.

We sampled the four remaining French populations, and selected three reference populations in Belgium, two of which are currently fragmented and declining but which used to be part of a large metapopulation until a few decades ago. The third is a relatively large and stable population.

We estimated the current and past demographic situation on the basis of genetic variation, the genetic diversity and the effective size of each population, and interpreted this in the light of recently developed genetic criteria to assess the local conservation status of populations.

Overall, our analyses show that all four French populations are genetically impoverished compared to Belgian populations, and that they have suffered stronger declines. None of the French populations can be considered to have a good conservation status for genetic criteria, which is mostly caused by small effective population sizes and small habitat size to sustain large enough populations.

We provide guidance for improvement of the conservation status and for future monitoring of these populations.

Recommendations for management and/or policy

Overall, we suggest the following actions:

1) Improve moor frog habitat quality and quantity where possible, in order to increase population sizes and reduce risks of inbreeding, and improve in general the conservation status of moor frogs in France
2) Improve the knowledge on the presence and spatial distribution of moor frog in N-France through eDNA research in habitats suitable for moor frogs
3) Explore the possibilities for genetic rescue through assisted gene flow
4) Monitor the existing populations closely by means of genetic and non-genetic methods

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1 INTRODUCTION

Among all vertebrate taxa, amphibians are the fastest declining on earth, with 32.5% of all species classified as globally threatened by the International Union for Conservation of Nature (IUCN) and 43.2% of species experiencing population declines. Over the past few decades, dramatic declines and extinction rates have been reported in Europe and North America (Houlahan et al. 2000), largely as a result of disappearance of wetlands. Their moisture dependability, breeding site fidelity and relatively low dispersal capacity render them vulnerable to habitat destruction, fragmentation and degradation (Smith & Green 2005).

The moor frog *(Rana arvalis*), is a widespread species in Eurasia that inhabits a wide range of lowland habitats with stagnant water bodies and littoral vegetation of low acidity. The ecological niche is distinct from *R. temporaria,* which prefers deeper water bodies and has a lower acid stress tolerance (Severtsov et al. 1998).

Except for the breeding season, when spawning and larval development takes place in stagnant water bodies, *R. arvalis* resides in terrestrial habitat within a typical home range of maximal 260 m^2 (Gyovai 1989; Loman 1984). Adults show lifelong terrestrial habitat fidelity and often return yearly to the same pond within 400m of their terrestrial habitat for spawning. After metamorphosis, the main long-distance dispersal event takes place when juveniles leave the water and disperse up to two km to colonise their terrestrial habitat (Pontoppidan & Nachmann 2013).

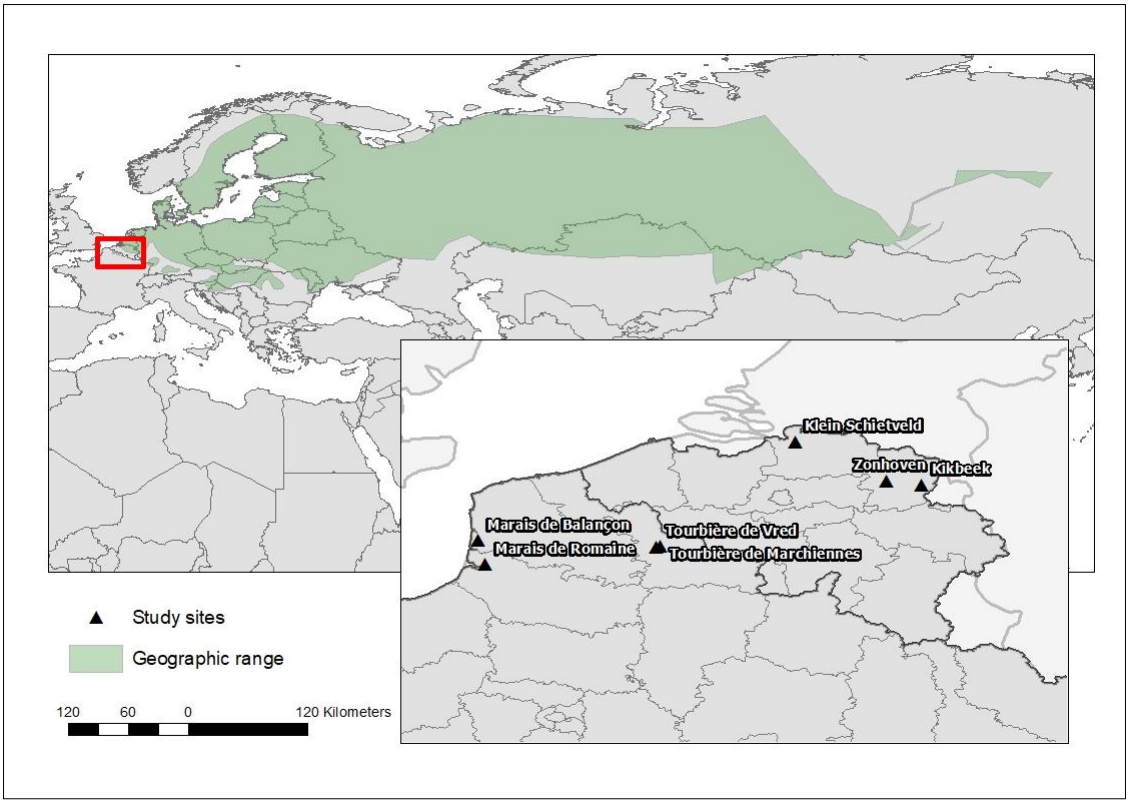

Figure 1: Map of the geographic range of *R. arvalis* and the departments/provinces in France and Belgium with officially documented *R. arvalis* populations marked in green. Adapted from www.iucnredlist.org.

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Figure 1 displays the geographic distribution of *R. arvalis*, showing that the western range edge is situated in N-France.

1.1 *R. ARVALIS* POPULATIONS: TRENDS AND THREATS

Although categorized as "Least Concern" on a global and European level on the IUCN Red List (Kuzmin et al. 2009), population declines of *R. arvalis* are reported throughout Europe. The main causes of population declines are destruction, fragmentation and degradation of habitat because of agricultural and urban development (Kuzmin et al. 2009).

At the end of the previous reporting period in 2013 for the European Habitats Directive, 17 out of 28 assessments of the European member states reported the species' habitat as not favourable, 9 of which with a deteriorating trend. 19 member states assessed the conservation status in total as unfavourable, 11 of which with a deteriorating trend (Appendix Table A1). The consistency in these results reveals the severity and substantial scale of the threats mentioned earlier, across many of the member states.

In Belgium, *R. arvalis* is nearly exclusively found in the provinces Antwerp and Limburg (Fig. 2). The species occurs in relatively large, continuous nutrient-poor habitats such as moorlands, swamps, moorland grasslands and moist forest with oligotrophic to mesotrophic water bodies. In smaller remnants of historic heathland, the species has largely disappeared (Colazza & Bauwens 2003; Jooris et al. 2013). Habitat area has been substantially reduced during the last century due to the disappearance of typical landscape dynamics associated with intense moorland management of the first half of the 20th century (Mergeay & Van Hove 2013). As a consequence, remnant populations have a rather fragmented distribution (Colazza & Bauwens 2003; Jooris et al. 2013). Due to this severely fragmented geographic range, and simultaneously observed declines in habitat quality, the moor frog is classified as vulnerable on the Flemish Red List (Jooris et al. 2013).

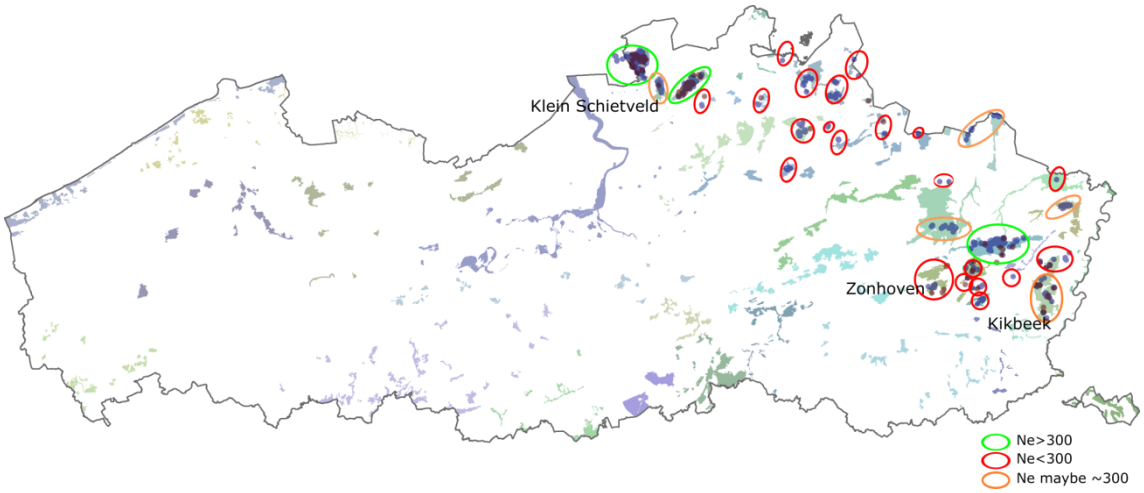

Figure 2. Distribution of moor frogs in Flanders with indication of the expected effective size on the basis of habitat quality and quantity (modified from Mergeay & Van Hove 2013). Background colours indicate location of Natura2000 areas.

In France, the moor frog's recent distribution is limited to four isolated localities in the departments of Pas-de-Calais, Nord and Somme. Its total area of occupancy is estimated to be slightly larger than 10 km^2 and population sizes are estimated to be over 250 (UICN-France et al. 2015). In the regional red listings, the species is classified as critically endangered. Because of this extremely small and decreasing number of populations and area of occupancy, as well

as the declines of populations and habitats, the species is classified as endangered on the Red List of France (UICN-France et al. 2015).

Apart from the long term unsustainability of the evolution towards increasingly fragmented distribution patterns, declining habitat quality poses a more immediate local threat for the survival of populations. Besides pollution, eutrophication and acidification of breeding ponds caused by agricultural activities, droughts and invasive alien species directly endanger the local survival of particularly the small populations in Belgium and France. In Belgium, the negative effects of acidification are exacerbated by associated Chytrid fungus infections that reduce egg survival (Jooris et al. 2013; Speybroeck & De Knijf 2019).

These threats and trends raise serious concerns about the condition and the future persistence of these particularly isolated range-edge populations. The virtual extinction in the highly cultivated landscape in the Alsace region (Vacher 2010) and the species' absence in more Western regions suggest that these concerns are justified and should be taken seriously.

1.2 THE IMPORTANCE OF GENETIC DIVERSITY IN BIOLOGICAL CONSERVATION

The Convention of Biological Biodiversity (CBD; United Nations, 1992) defines genetic diversity as the within-species component of biodiversity, which is the foundation of the species- and the ecosystem diversity components. Genetic diversity is highly relevant in the current biodiversity crisis because the survival and adaptive potential of species, and thus ultimately ecosystems, depend on it. The loss of genetic diversity (genetic erosion) increases extinction risk because its leads to lower average fitness and a decreased ability to adapt to changing environments and cope with anthropogenic pressures (Frankham 2005).

Genetic erosion is both a result and driver of population declines, and generally precedes the extinction of a species. As a consequence, the rate of genetic diversity loss is likely to be even more alarming than the rate at which species diversity is lost. 70% of natural populations is estimated to lose genetic diversity at a rate that threatens their long term persistence. Species of conservation concern are found to exhibit even stronger reduced levels of genetic diversity (Frankham 2005; Palstra & Ruzzante 2008).

1.2.1 Measures of molecular genetic variation

Molecular population genetic parameters directly reflect genetic variation in the genome by quantifying the variation and patterns in genotypic and allelic frequencies. Because this branch of population genetics mainly focuses on neutral diversity, it is well suited for studying neutral processes such as genetic drift, inbreeding, and gene flow (Hamilton 2009).

The most widely used molecular population genetic parameters that quantify the amount of standing genetic diversity are the allelic richness (AR), the gene diversity (aka expected heterozygosity, H_e) and the effective population size (N_e). The allelic richness reflects how many alleles are present at each locus for a given sample size. The gene diversity takes into account frequency differences among alleles. The effective population size (N_e) is a parameter that reflects the genetic drift (random loss due to a finite size) a population experiences per generation. In Ideal Wright-Fisher populations, population size (N) is the only determinant of genetic drift. In absence of all other evolutionary forces, such a population loses 1/2N of its H_e per generation. In real populations however, size fluctuations, founder events, variance in reproductive success, non-random mating or unequal numbers or reproductive success of

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males and females all lead to increased drift. Because N_e captures how fast a population is losing genetic variation as well as the contemporary degree of inbreeding, it allows prediction of its vulnerability and long-term viability (Wang et al. 2016).

1.2.2 Threats to genetic diversity

Loss of genetic diversity has effects on the evolutionary potential of the population and on fitness through inbreeding depression. The first is indispensable for the long term survival of species while the latter has a short term impact on extinction risk (Frankham 2005).

Since habitat loss and fragmentation and degradation cause population sizes to progressively decline, they go hand in hand with drift and a loss of genetic diversity. Consequently, the remaining populations increasingly experience negative genetic effects of inbreeding and loss of evolutionary potential (Ralls et al. 2018). These factors moreover enhance each other through positive feedback loops.

Across population fragments, gene flow can play an important role, by compensating the local loss of genetic diversity through genetic drift by replenishing genetic variation to the subpopulation from other subpopulations. As a result, genetic connectivity will cause subpopulations to lose genetic diversity at a pace determined by the N_e of the total metapopulation rather than the N_e of the subpopulation. In general, it is considered that one migrant per generation (OMPG) per subpopulation is required to mitigate the effects of local genetic drift sufficiently. Very often, however, such connectivity is hard to achieve in highly fragmented landscapes, such as those of central Europe (Jaeger et al. 2011; Palstra & Ruzzante 2008).

1.3 GENETIC CRITERIA FOR FAVOURABLE MOOR FROG CONSERVATION

Mergeay (2013) developed a set of genetic criteria to evaluate the genetic status of populations relative to a favourable reference value (FRV) at three hierarchical spatial scales: local populations, embedded in a metapopulation, which are themselves relatively independently evolving units relative to the total population in a given biogeographic area.

The total population requires an effective size that exceeds a N_e of 1000 or a N_c of 10 000 (assuming a 1/10 N_e/N_c ratio; Frankham 1995). At this population size, the mutation frequency is expected to compensate for the loss of genetic diversity due to genetic drift (Frankham et al. 2014 Frankham et al. 2014). The FRV for a local (meta)population reflects the minimal N_e required to retain 95% of its genetic diversity by drift over a time span of 100 years, which is called Ne95 (Mergeay 2013). This is calculated by solving N_e in $N_e \approx \frac{-t}{2Ln(\frac{H_t}{H_0})}$, for $\frac{H_t}{H_0} = 0.95$ and t=(number of generations per 100 y).

The absolute value of Ne95 depends on the amount of generations within this timeframe, and will therefore be higher for species with short generation times. Populations are considered to be part of a metapopulation if they are functionally connected by at least one effective migrant per generation (OMPG) (Mills & Allendorf 1996). Populations not part of a metapopulation can only have a FRV if they are larger than the Ne95. Appendix Figure A1 provides more information.

For the moor frog, the estimated generation time is 3.3 years (Gyovai 1989), which translates into a Ne95=295, or a census size of approximately 2950 adults. Assuming an average density of 20 adults/ha in good habitat in W-Europe (Alterra 2001), this translates into a required area

of nearly 150 ha, which should contain of a mosaic of terrestrial and aquatic habitats detailed in Appendix Table A2. In practice, the species can be found in a wide range of terrestrial habitats (grasslands to forests) (Drobenkov et al. 2005), but it seems associated to water bodies with a low pH (<6) (Brys et al. 2020; Nöllert & Nöllert 2001), as a result of which terrestrial habitats in W-Europe are typically associated to moors and heathlands. The highest recorded densities of this species are achieved in Eastern European mesotrophic to oligotrophic floodplain forests, where densities average 340 individuals per ha (Drobenkov et al. 2005). Such densities, however, are unlikely to be achieved in W-Europe.

Local assessment of the Ne95 criterium can be done directly by conducting molecular population genetic research to derive N_e estimates and compare them to the FRV of Ne95. Indirect assessment is possible through census size estimates if the N_e/N_c ratio is known. Often, this ratio is assumed to be 0.1 (Frankham 1995), but this can vary across species.

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2 GOALS OF THE STUDY

In this study, we want to evaluate key parameters of genetic diversity in the four remaining French populations, and compare these to three Belgian populations of known status. Two of the French populations are close neighbours, and may still exchange migrants occasionally. Specifically, we will determine genetic variation at 15 microsatellite loci in each population, and use these results to

1. compare metrics of genetic diversity among populations
2. estimate genetic differentiation among populations
3. estimate gene flow among the two neighbouring French populations
4. estimate the effective size of each population
5. detect which populations underwent measurable genetic bottlenecks
6. compare the effective size to genetic criteria for sustainable conservation

3 METHODS

3.1 STUDY SITES

In France, the four known remaining populations were studied. Two of them are situated in "Parc Naturel Régional Scarpe-Escaut" in the Département du Nord: Tourbière de Vred and Tourbière de Marchiennes. The others are located at Marais de Balançon in the Département de Pas-de-Calais and at Marais de Romaine in the Département de Somme (Fig. 2). Currently, these are the only known populations in France, representing the western edge of the distribution of this species (Blondel 2014; Vacher 2010). In Belgium, three sites were selected: Klein Schietveld in the province of Antwerp, and Kikbeek and Zonhoven in the province of Limburg. These populations serve mainly as references of a currently large and two medium to small populations, respectively, for comparison with the French populations. Detailed maps of each locality can be found in Appendix Fig. A2 to A7.

We will further briefly summarise the habitat condition and landscape history of the location each population occurs in.

3.1.1 Populations in Belgium

Klein Schietveld comprises a nearly 900 ha area, of which 75 ha is wet moorland, mostly situated in the Northern part. Stagnant acidic waters are plentiful (10 ha) in this part. It was safeguarded from peat extraction and other types of exploitation that occurred widely throughout the 19th century because of its function as a military training area. Now however, habitat quality is compromised by the exceeding nitrogen deposition limits throughout the entire area. Water quality of the fens is still good (De Saeger et al. 2018). The neighbouring area 'Groot Schietveld' is the largest continuous potential habitat for *R. arvalis* in Belgium. On the Ferraris map (1777), both were still connected. Because of its high relative proportion of total habitat area and/or high habitat quality the 'Schietvelden' are one of three Natura2000 sites that are classified as essential for moor frog (Paelinckx 2009). With an estimated total area of suitable and occupied moor frog habitat of 109 ha, this population is considered close to the Ne95 criterium of 150 ha.

The Valley of the **Kikbeek** source is a former sand quarry pond with heath vegetation. It is part of a The Natura2000 site 'Mechelse Heide en Vallei van de Ziepbeek'. Historically, this area used to be a river valley with fens and moist heath, connected with the more Northern parts of the Natura2000 site. During the beginning of the 20th century, forest area increased until 1930 after which heath vegetation gained area again, until the establishment of the quarry in 1961 allowed only a few ha to persist in the East. In the 1970s it was separated from the Mechelse Heide due to construction of the E314 highway. In 2005, an ecological connection was re-established through the establishment of a wildlife passage (ecoduct Kikbeek).The exploitation ceased in 2004 and recently, the former natural river bed has been restored and some small fens and moist heath vegetation have returned. Although this area is a is a part of the National Park Hoge Kempen with several *R. arvalis* populations, they each occur in fragmented fen clusters without intermediate habitat, at distances of 2 km or more from each other. The total area of occupied habitat is estimated at 56 ha, though more sparsely populated than Klein Schietveld.

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The third population is situated between Zonhoven and Bolderberg, within the 719 ha Natura2000 site subregion “Vijvergebied Midden-Limburg”. We will further refer to it as **Zonhoven**. The area has an extensive history of utilization for peat extraction and fishing combined with extensive agriculture. The current landscape was already as such on the Ferraris map (1777). However, at this time heathland was still abundant, after which it progressively disappeared, at first due to the intensification of fish farming. Later, during the second half of the 20th century, heathlands further disappeared by conversion to meadows for grazing, causing many fishing ponds to disappear as well. After 1950, heathland area decreased even more due to industrialization and population growth (Mergeay et al. 2018). Currently, eutrophication of the dozens of ponds is an issue in this area. This can be attributed to the poor quality of the rivers that serve as water sources for the ponds. A dozen of *R. arvalis* (sub)populations are known to be present but their connectivity remains unknown. Mergeay & Vanhove (2013) concluded that the different subpopulations in this region are too fragmented to meet the Ne95 criterium. Moor frogs occur over an area of circa 90 ha, clustered along six or seven ponds, which are each within dispersal distance of each other.

3.1.2 Populations in France

In the Scarpe-Escaut Parc Naturel Régional, moor frogs were deemed extinct, but populations were rediscovered in 1999 at two locations. **Tourbière de Marchiennes** and **Tourbière de Vred** are two protected sites of 66 and 55 ha respectively, circa 3 km apart. Both are classified as ’Zone naturelle d'intérêt écologique, faunistique et floristique’(ZNIEFF). They consist of mainly alkalic peat bogs, which were historically used for peat extraction, many of which have been converted to other land uses. Only a few ha of remnant acidic oligotrophic fens still serve as aquatic moor frog habitat. Historical maps indicate that moorland was plentiful in the area (> 10 000 ha) during the 18th century (Fig. 3).

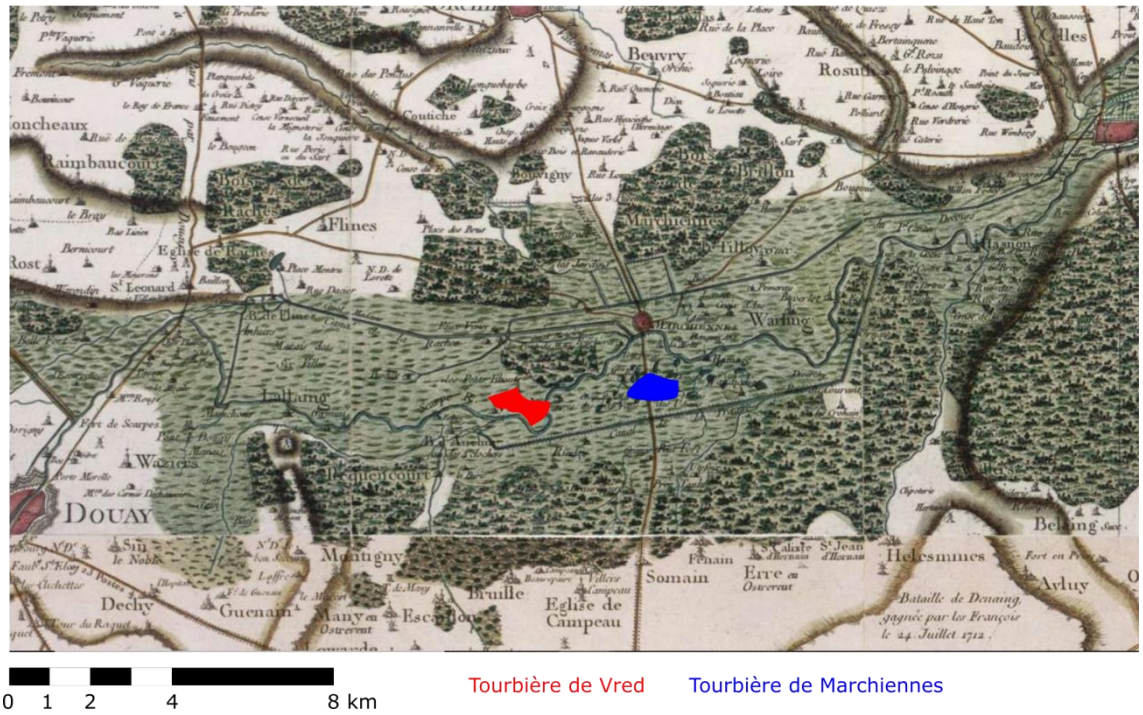

Figure 3: Historic map (https://www.geoportail.gouv.fr/donnees/carte-de-cassini) of the marshlands and forests in the area of Tourbières de Vred and Marchiennes in the 18th century.

The two current population remnants were certainly part of a large continuous metapopulation situated in the marshlands and floodplains of the Scarpe river, with extensive peat extraction sites (tourbières) across the area. Currently, gene flow among these two populations is questionable, being separated by the Scarpe river, two roads and agricultural land (Rondel et al. 2013). Effective reproduction of *R. arvalis* was confirmed in 2003, after which considerable effort was put into providing favourable habitat for the species and into general ecological and hydrological restoration Rondel et al. 2013). From 2008 to 2011, the condition of the population and their potential habitat was assessed, resulting in an estimate of 39 and 16 occupied ponds in Tourbière de Marchiennes and Tourbière de Vred respectively. No more than 14 to 17 and 21 to 28 calling males were counted per site, respectively (Rondel et al. 2013).

In **Marais de Balançon**, a moor frog population was only recently discovered in 2011. It is a 1007 ha Natura2000 site consisting mainly of marshes (70%) and fens and stagnant waters (20%) (Bigot et al., 2013). It also is a 784 ha ZNIEFF site, for a large part overlapping with the Southern part of the Natura2000 site. An area of 180 ha of the latter is considered to be suitable habitat for *R. arvalis,* mainly situated in the ZNIEFF site (Bigot et al., 2013). Moor frogs are estimated to be present throughout at least 50 ha of this area (Benjamin Blondel, Pers. Comm.). However, this has not always been the case. Construction of drainage ditches in the marsh started as early as the 16th century in order to facilitate the establishment of human communities. Peat extraction started at the end of the 18th / early 19th century and lasted up to 1930, when coal deposits in Pas-de-Calais were discovered and peat exploitation was quickly marginalized. Subsequently, large-scale drainage of the marshes enabled agricultural practices until agricultural intensification after 1950 gradually allowed the abandonment of the wettest areas, allowing the marshes to return. The main activity then became, and still remains, hunting. To date, very little is known about the present *R. arvalis* populations. However, several management objectives were set and are being applied for the benefit of *R. arvalis* populations (Bigot et al. 2013).

The fourth moor frog population was only recently discovered in 2014 in the department of Somme at **Marais de Romaine**, Ponthoile (Blondel 2014). This population is situated 20 km North of Marais de Balançon. Inspections for the species have been conducted in the surrounding areas, but without success so far. It is noteworthy that many sites are not accessible and are private land. Marais de Romaine is located within the 1623 ha Natura2000 site “Marais arrière-littoraux picards”. Within this site, restoration management to restore natural abiotic conditions have been going on and additional practices have been put in place to favour the establishment of the species in a wider area within the site (Blondel 2014). The protected part of Marais de Romaine itself has an area of 56 ha, of which c. 4 ha consists of Natura2000 habitat types typical of *R. arvalis*. However, the total amount of suitable habitat is likely much larger, as there seems to be ample aquatic and terrestrial habitat types typical of moor frog (Dufour & Triplet 2009: annexe III; Bigot et al. 2013), also outside the protected area.

3.2 SAMPLING AND MOLECULAR ANALYSES

Either adults were caught during targeted visits between March and May and sampled with a buccal swab, and a single egg was taken from egg clumps in March and April. In France, buccal swabs were sampled by French volunteers in the framework of this study, and were available from consecutive years (2016, 2017, 2018, or 2018 and 2019 for Marais de Romaine). (Appendix Table A3 provides details of sample sizes per site and per year). Swabs were either air-dried for several hours after which they were stored in a cool dark place for short-term storage, either immediately stored at -20°C for long-term storage.

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In Kikbeek and Zonhoven, 60 and 42 egg samples, respectively, were taken from a single fen (one per clutch). Egg sampling in Klein Schietveld was performed in the framework of a different study, involving detailed landscape-genetic analyses of moor frogs (Brys et al. 2020). Here we use a subset of 172 genotypes from 19 fens genotyped by Brys et al. (2020). Adult samples were taken over an area of 1 ha in the central part of the area on a single night. In Tourbières de Vred and Marchiennes, sampling occurred over a total sampling area of 0.9 and 3.2 ha within the total ZNIEFF area sizes of 55 and 66 ha, but which covered the entire area of known occurrence. In Marais de Romaine, adult sampling occurred over an area of 8.5 ha. In Marais de Balançon, an area of 6 ha within the central part of the total ZNIEFF area of 784 ha was sampled in the centre of the species' range of occurrence.

3.2.1 DNA extraction

DNA from eggs was extracted by first removing the outer jelly membrane of each egg with a scalpel. Next we used the Blood & Tissue Kit (Qiagen, Venlo, the Netherlands) with a lysis step of 1 hour. The DNA was eluted in 70 µl AE buffer (elution performed twice).

DNA from swabs was extracted by cutting off the cotton matrix from each swab. Next, we used the QiaAmp Micro Kit (Qiagen) with a 1h lysis step, and elution of DNA in 50 µl AE buffer. The integrity of DNA of 10% of the samples was assessed on 1% agarose gels. DNA quantification was performed with Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies) using a Synergy HT plate reader (BioTek).

3.2.2 Molecular markers

Since egg clutches of moor frog occur often together with clutches from common frog (*Rana temporaria*) which look very similar, they need to be distinguished genetically. We used the method of Palo and Merilä (2003) using the same step and conditions described in Brys et al. (2020). In short, this uses restriction fragment length polymorphism to amplify part of the mitochondrial DNA (cytochrome b) and distinguish both species on the basis of the restriction pattern using agarose gel electrophoresis.

We amplified 19 microsatellite markers, of which eventually 15 were retained for population-genetic analyses (see Brys et al. 2020). The list of markers and PCR cycling conditions can be found in Appendix Table A4. Genotyping was performed on a ABI3500 Genetic Analyzer (Applied Biosystems), with an internal LIZ600 size standard. Allele calling was done with Genemapper 6.0 software (Applied Biosystems).

3.3 DATA ANALYSES

3.3.1 Genetic variation

Analyses involving R packages were performed in R 3.6 (R_Core_Team 2018) and RStudio (RStudio_Team 2020). To test the data quality, we used MicroChecker (Van Oosterhout et al. 2004) to detect loci with large allelic dropouts and scoring errors. GENEPOP v4.0 (Rousset 2008) was used to test for null alleles and deviations from Hardy-Weinberg equilibrium that may be indicative of other violations of population genetic model assumptions. We used the R Package Hierfstat 0.0.4-22 (Goudet 2005) to calculate gene diversity (expected heterozygosity, H_e), allelic richness (AR, corrected for sample size) and F-statistics. Since the different loci have varying absolute diversity levels, we standardized allelic richness and gene diversity H_e per site per locus to the maximum observed value in any location. We then test if average and standard deviation of AR and He per site differ among populations, using ANOVA. We use post hoc Tukey HSD tests to evaluate which sites display pairwise significant differences. The

significance of departures from HWE was tested with the Markov Chain method to estimate p-values for the U test, implemented in GENEPOP.

3.3.2 Genetic structure and gene flow

Genetic structure was assessed using estimates of pairwise D_{est} (a metric that describes exact allelic differentiation) and the fixation index F_{ST} (or actually, the multivariate unbiased estimate derived from it, G_{ST}). Significance of pairwise differences was tested with 999 permutations using R package Hierfstat 0.0.4-22 (Goudet 2005). F_{ST} is a widely used metric to establish genetic differences, but it is not actually a differentiation metric: it measures how close populations are on average to fixation (loss of genetic diversity). D_{est} provides an exact allelic differentiation, but it is more difficult to interpret in terms of which processes (mutation, migration, genetic drift, ...) led to the observed differences.

PCoA (a multivariate linear ordination technique) was performed on the basis of pairwise Nei's genetic distances (among populations) and of euclidean distances (among genotypes, yielding a PCA). From genotype-based PCA, we performed discriminant analyses of the resulting principle components (DAPC, a method to cluster genotypes so within-group variance is minimized and among-group variance is maximized). These analyses were performed using the R-package Adegenet 2.1.0 (Jombart 2008). DAPC groups were pre-defined as the sampling locations. The number of PC-axes to retain for the DAPC was based on the a-score (Jombart & Collins 2015).

3.3.2.1 Self-assignment and membership probabilities

Since populations were clearly sampled discretely in space and the true genetic clusters are therefore known, we tested to what extent we had sufficient genetic resolution to assign each population to its true origin, using a self-assignment procedure in Geneclass 2 (Piry et al. 2004). We used the Bayesian criterion of Rannala & Mountain (1997) and a threshold of 0.05. This provides for each individual genotype a likelihood assignment score for each of the populations of origin. A high overall score indicates a high resolution to detect genetic differences among populations.

3.3.2.2 Gene flow estimates between Vred and Marchiennes

The calculation of contemporary migration rates (the proportion of migrants between populations) was done in BAYESASS with default parameter settings (Wilson & Rannala 1997). This analysis was restricted to Vred and Marchiennes, with samples pooled over the three sampling years (samples pooled over years). We also used Geneclass2 for the detection of first-generation migrants between Tourbières de Vred and Marchiennes using the frequency based criterion of Paetkau et al. (2004). Statistical power of this test was assessed visually through the distinctness of the log-likelihoods clusters obtained for samples of both populations. This test provides for each genotype a -log likelihood score for each possible source population (including its home population), and calculates the log of the ratio of the highest score to the score of its home population. When an individual is assigned to its own population, the resulting score is zero, when it is not, the score is larger than zero.

To estimate the degree of false positive estimates of gene flow, the same Geneclass2 analysis was also run on the complete dataset, where the absence of any level of migration is a priori known. We compared the log likelihood ratio values Log(L-max/L-home) to estimate to what extent our analyses are robust overall.

3.3.3 Effective population size

We estimated N_e on the basis of gametic disequilibrium (linkage disequilibrium) using LDNe (Waples & Do 2010). This estimates the inbreeding population size of the parental generation. When single cohorts are sampled in a species with overlapping generations (such as the moor frog), this estimates the effective number of breeders (N_b) rather than the effective size (N_e).

Alleles with a frequency lower than 0.02 were excluded from the analysis. This is advised when sample sizes are moderate (<50) and consequently the estimation of rare allele frequencies is prone to sampling errors that inflate linkage disequilibrium. When applying the method to the single cohort samples in Belgium and to the mixed-cohort adult samples taken in the French populations and in Klein Schietveld, N_b and N_e were estimated, respectively.

Estimates of N_b can be transformed to N_e estimates according to Waples et al. (2014), as a function of three life history traits: age at maturity (α), adult life span (AL), and an index of variation in age-specific fecundity (CVf). These values were obtained from a life table with age-specific survival probabilities and fecundities (Pontoppidan & Nachmann 2013) (see Appendix Table A5). Because of uncertainty concerning the duration of the adult life span, a minimum and maximum adjusted N_b were calculated on the basis of minimum and maximum values of 3 (Gyovai 1989) or 6 (Fog & Hesselsøe 2009) for this variable. After this, N_e was calculated as a function of the adjusted N_b and the three life history traits.

In Klein Schietveld, N_e was estimated separately using eggs sampled across most of the area (N=172 in 2017) on the basis of the bias adjusted estimate of N_b, and using adults (N=31 in 2019). The former was done to increase coverage, the latter to represent a more typical sample, in size and age composition, used in conservation genetics studies and to obtain more comparable estimates as to those obtained for the French populations.

Where mixed-cohorts were sampled, which was the case for Klein Schietveld (adults sampled in 2019) and for all French population over all years, the LD method directly estimated N_e. For the French populations, where multiple consecutive years where sampled, the method was applied on both the per-year samples as well as on the mixed-years data per population.

In Tourbière de Vred, Tourbière de Marchiennes and Marais de Balançon, adults were sampled over 3 years (2016-2018). In Marais de Romaine, adults were sampled in 2018 and 2019. This allows the N_e to be estimated using a second, temporal method as well. More specifically, we used a maximum likelihood method implemented in the R package NB (Hui & Burt 2015), with a maximum prior N_e of 10000. This method did not account for sampling intervals lower than one generation, as was the case with our data that were obtained over consecutive sampling intervals of 1/3th of a generation. However, a linear relationship exists between the length of the sampling interval and estimated N_e and the calculated upper and lower confidence interval (CI) bounds, while log-likelihood values remain identical. Hence, we performed the analyses with a sampling interval of one generation, after which we divided the resulting N_e and CI bounds by three.

3.3.4 Genetic bottlenecks

We used Bottleneck v. 1.2.02 (Cornuet & Luikart 1996) to test if the populations show significant deviations from mutation-drift equilibrium. At mutation-drift equilibrium, the number of alleles in a sample can be used to predict the gene diversity. Since we know the number of alleles and we can measure the actual gene diversity, we can test if it is significantly larger or smaller than the predicted gene diversity. When a population undergoes a contraction (bottleneck), the allelic richness is very rapidly reduced, whereas the response of

the gene diversity is delayed in time. Likewise, a population expansion will lead to the occurrence of new (rare) alleles and increase the allelic richness through mutation, whereas gene diversity takes a long time to recover. This difference in response times between allelic richness and gene diversity to population size changes can be exploited to detect signals of historical population change. Mark that genetic bottlenecks can also result from loss of gene flow among neighbouring populations (Broquet et al. 2010).

A larger than expected gene diversity indicates that the population went through a bottleneck and will continue to lose gene diversity until a new lower equilibrium is reached. A smaller than expected gene diversity indicates that the population underwent a population expansion. Per population, genotypes sampled across years were pooled to increase sample size. This is not an issue as the method does not rely on Hardy-Weinberg expectations. We used the infinite allele model, as this model does not assume that alleles originated independently in each population, and is better suited to detect recent rather than ancient demographic changes. Significance (one-tailed tests for gene diversity excess and gene diversity deficiency) was tested by means of the Wilcoxon test (1000 replications) implemented in Bottleneck. Effect sizes were calculated by taking the average of the DH/SD statistic, which represents the difference of the actual gene diversity and the predicted gene diversity, divided by the standard deviation. The higher this absolute value, the stronger the deviation from equilibrium is.

This method provides no detailed information on the timing of the bottleneck or expansion. This would require specific simulations of various population scenarios, outside of the scope of this study.

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4 RESULTS

4.1 GENETIC DIVERSITY

4.1.1 Data quality

All PCR replicates (n=25) yielded identical genotypes, resulting in a 0% genotyping error estimate. Genotypes with missing data at more than 4 loci were removed from the analyses. Overall, this yielded a total of 537 unique genotypes. Locus RlatCa41 yielded significant deviations from HW equilibrium in populations Kikbeek, Klein Schietveld and Zonhoven, but not in Marchiennes and Vred. In Balançon and Romaine this locus was not polymorphic. Genepop estimated the frequency of null-alleles at this locus in Kikbeek, Klein Schietveld and Zonhoven to be 15.3% (95CI 7.6%-23.3%), 16.7% (95CI 12.6%-20.8%) and 7% (95CI 0.0%-9.7%). No other major indications for null-alleles were observed across the dataset. We omitted locus RlatCa41 for analyses sensitive to deviations from HW-equilibrium and linkage disequilibrium. We retained the locus for other analyses.

4.1.2 Polymorphism, allelic richness, gene diversity

Population Kikbeek was the only to be polymorphic at all fifteen loci. In decreasing order and with number of polymorphic loci between brackets, the levels of polymorphism were: Kikbeek (15/15), Klein Schietveld (14/15), Zonhoven, Marchiennes (13/15), Romaine (12/15), Vred, Balançon (11/15). Kikbeek also showed the highest mean allelic richness (AR) and gene diversity (H_e) (Table 1).

Table 1. Genetic diversity summary statistics per population, over all 15 microsatellite loci. AR: allelic richness; H_e: gene diversity (expected heterozygosity). SE: standard error.

	Balançon	Romaine	Marchiennes	Vred	Kikbeek	Kschietveld	Zonhoven
Mean AR	3.177	3.368	4.569	3.672	6.603	5.522	5.831
SE	0.526	0.531	0.734	0.589	1.220	0.995	1.061
Mean H_e	0.407	0.391	0.507	0.489	0.582	0.533	0.531
SE	0.078	0.079	0.067	0.082	0.068	0.073	0.083

The standardized allelic richness differed significantly across populations (AR: ANOVA $F(6,98)=11.87$, $p=5.67 *10^{-10}$), and so did standardized gene diversity (ANOVA $F(6,98)=2.406$, $p=0.0327$). The Tukey Test results showed that except for Marchiennes, all French populations showed significantly lower standardized allelic richness than any of the three Belgian populations. Marchiennes only showed a significantly lower allelic richness than Kikbeek, the richest of all populations. Among the Belgian populations, there were no significant differences, nor among French populations. For standardized gene diversity, the Post-hoc test only indicated a pairwise significant difference between the two poorest populations (Balançon p=0.05 and Romaine p=0.03) and the most diverse population (Kikbeek). All other pairwise comparisons were non-significant.

On average, however, allelic richness was 38% higher in Belgian populations than in French populations (t-test: t=-5.05157, p=0.002). Likewise, the effective number of alleles (calculated as $1/(1-H_e)$) was on average 18% higher in Belgian populations (t-test: t= -2.92781, p=0.016).

4.1.3 Genetic structure

4.1.3.1 Genetic distances among populations

Pairwise genetic distances are shown in Table 2. All pairs of populations show pronounced and significant genetic differentiation ($p \leq 0.001$ for 999 permutations), for D and G_{ST}. Both metrics showed similar overall results, even though G_{ST} was always lower. The lowest differentiation was found between Zonhoven and Klein Schietveld, despite the considerable spatial distance (80 km) between them. The highest differentiation was observed between Balançon and Marchiennes.

Table 2. Pairwise genetic distances among the seven populations. Above the diagonal the fixation index G_{ST} is shown, below the diagonal D_{est} is shown.

	Balançon	Kikbeek	Kschietveld	Marchiennes	Romaine	Vred	Zonhoven
Balançon		0.168	0.134	0.214	0.114	0.170	0.138
Kikbeek	0.183		0.087	0.174	0.188	0.191	0.092
Kschietveld	0.144	0.091		0.128	0.187	0.125	0.074
Marchiennes	0.241	0.191	0.137		0.200	0.109	0.152
Romaine	0.121	0.208	0.207	0.224		0.162	0.182
Vred	0.187	0.211	0.133	0.115	0.177		0.144
Zonhoven	0.148	0.096	0.077	0.165	0.201	0.155	

4.1.3.2 PCoA

In the PCoA on Nei's genetic distances (between sampled populations), the first two axes explained 61.4% of the genetic variance (Fig. 4). The first axis separates Romaine and Balançon from the other populations, whereas the second axes further separates between the three Belgian populations and the two populations from Scarpe-Escaut, Marchiennes and Vred. Overall, we can infer three major clusters of populations corresponding to geographic origins.

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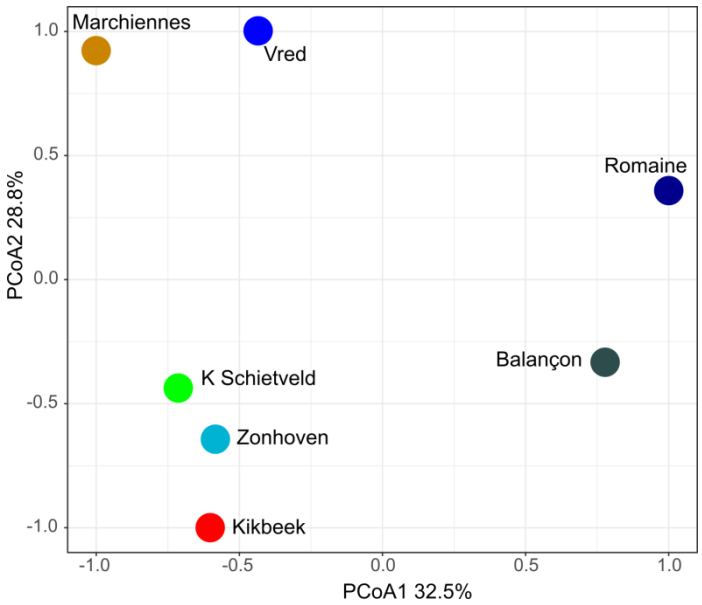

Figure 4. PCoA-plot of the first and second PCo-axes (with eigenvalues standardized to the range -1 to 1), on Nei's genetic distances among populations (samples pooled across years). The plot clearly shows the high genetic proximity of the three Belgian reference populations, relative to the French populations.

4.1.3.3 DAPC

A DAPC on the existing populations as prior groupings was performed on the basis of the first 40 PCA axes, which represent 83% of the total variance in the dataset.

The first four DA axes were retained, and graphically represented in two plots (Fig. 5). These plots (with inertia ellipses around the population centres) show a similar pattern as the population-based PCA, but moreover show the spread of individual genotypes (dots connected to the population centres) when the within-group variance is minimized. Each population is clearly separated from any other population by a combination of these four axes. Even the closely situated and related populations of Marchiennes and Vred show little overlap in their inertia ellipses along DA3 and DA4. Zonhoven shows most overlap with both Kikbeek and Klein Schietveld, whereas the latter two are clearly separated along DA3.

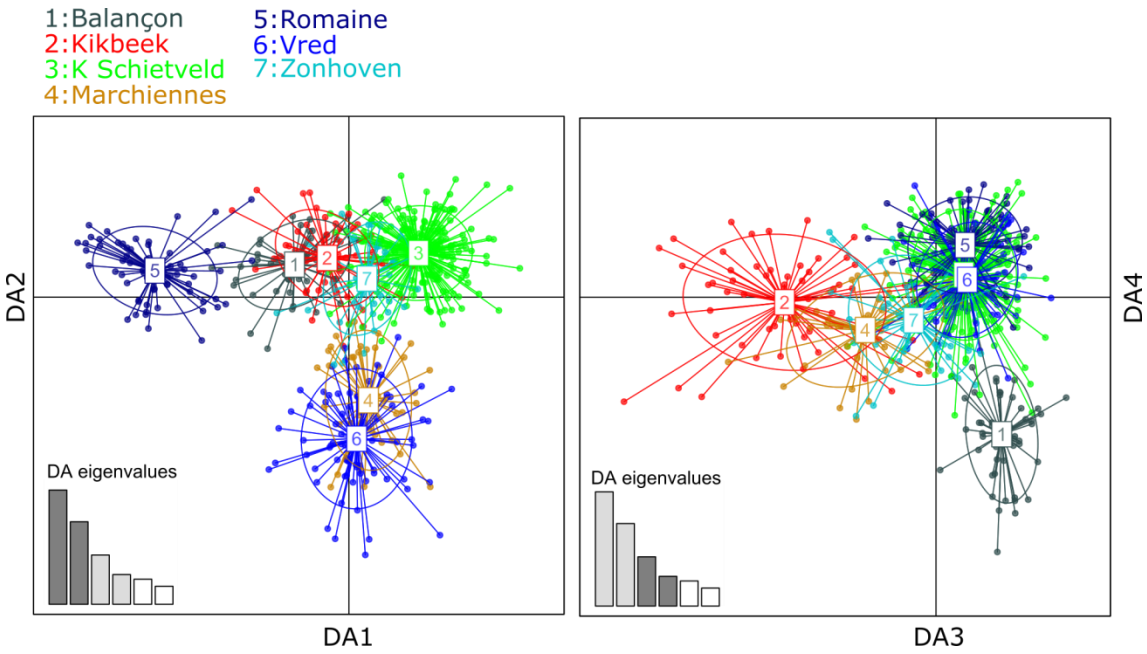

Figure 5. DAPC plots of linear discriminant axes 1 and 2 (left) and axes 3 and 4 (right). Colours indicate the population to which each genotype belongs. The population centre is indicated by a number, individual genotypes are dots connected to the population centre. 95% inertia ellipses represent graphical summaries of a cloud of points, and can be viewed as confidence zones. The combined graphs show the relative genetic nearness of populations along various axes.

4.1.3.4 Self-assignment

The overall quality index of the Bayesian self-assignment in Geneclass 2 was 97.3%. Overall 527 out of 537 genotypes (98%) were correctly assigned to their original population. High self-assignment scores indicate clear-cut genetic clusters, while low scores are indicative of admixture or of recent common ancestry. Populations Balançon, Romaine, Vred and Zonhoven showed 100% self-assignment. Populations Kikbeek and Klein Schietveld had self-assignment scores of 95% and 98%, respectively. Marchiennes' self-assignment score was 88%. For Marchiennes, 4 out of 43 genotypes were assigned to the neighbouring population (Vred, with probabilities from 51% to 100%), while one was assigned confidently to Zonhoven. Overall, this indicates that all populations have undergone clearly separate neutral evolutionary trajectories.

4.1.3.5 Gene flow among Vred and Marchiennes

The only two populations for which we can reasonably assume among which there could be contemporary gene flow are Tourbière de Vred and Marchiennes, as they are just within the maximum dispersal distance of moor frog (3 km) of each other (Vos & Chardon 1998). Contemporary migration rates (the fraction of individuals exchanged per generation), calculated with BayesAass were 0.0054 from Tourbière de Vred to Tourbière de Marchiennes and 0.0332 the other way around. To compare this to the number of migrants, we should multiply with the effective size of each population. This yields Nm values well below the threshold of one migrant per generation.

Detection of first generation migrants with Geneclass2 performed on the mixed-years data of Tourbière de Vred and Marchiennes, using the frequency based criterion (Paetkau et al. 2004) led to the detection of 2 individuals sampled in Marchiennes that were assigned with high

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confidence (99.6% and 100% probability) as being first generation migrants originating from Vred. No other samples received a probability value below 0.01. Both genotypes corresponded to individuals sampled in 2018.

To assess the statistical power, log likelihoods were plotted for Vred and Marchiennes. Figure 6 displays two clear clusters of individuals assigned to the population they were sampled in. The two first generation migrants identified in Tourbière de Marchiennes are clearly situated centrally within the 'Tourbière de Vred' cluster.

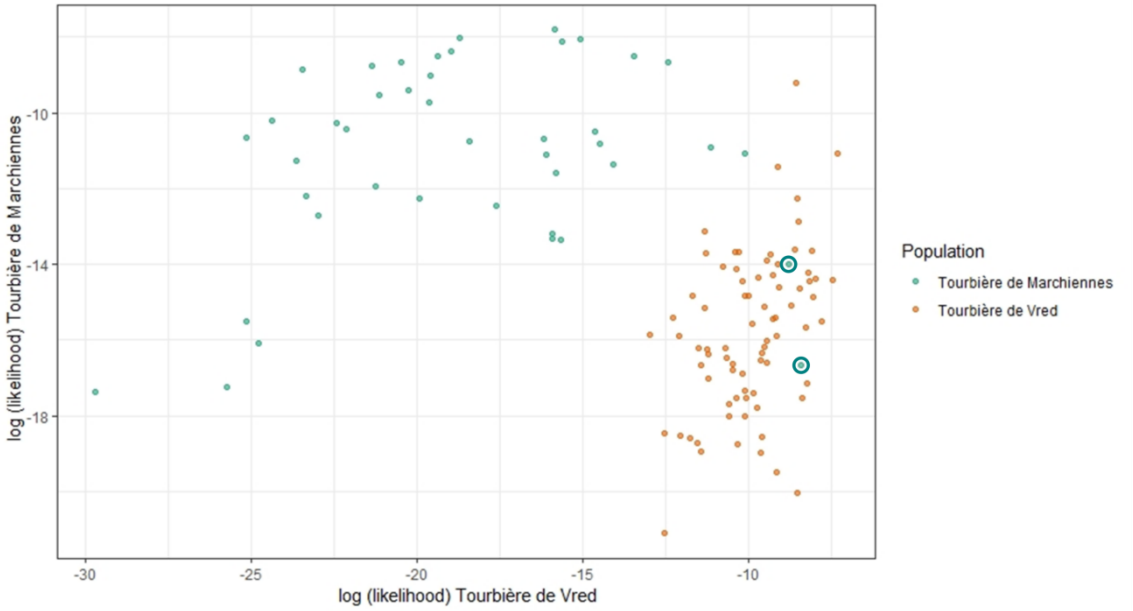

Figure 6: Log genotype likelihood of the individuals sampled in the populations of Tourbière de Vred and Marchiennes. The two first generation migrants identified by Paetkau et al.'s (2004) frequency-based assignment method are indicated. The mean distance from the centre diagonal is a measure for the statistical power of the test.

As a control, the same analysis was also run on the complete dataset. The individual from Marchiennes that was identified with the self-assignment as belonging to Zonhoven was not identified as a migrant from Zonhoven, but to its own population. In the total dataset, 18 additional individuals showed positive scores of the Log likelihood ratio (L-home/L-max). The scores of the two supposed migrants from Marchiennes to Vred were, however, much higher (5.3 and 8.8) than the remaining scores (average: 0.85, range 0.11 – 2.55).

4.1.4 Effective size estimates

We used two approaches for estimating effective sizes: we first used a temporal method that measures the genetic change among generation due to genetic drift. Second, we used a method based on linkage disequilibrium. The first method was only available for the French populations, as these were the only ones to have been sampled over multiple years.

4.1.4.1 Temporal method

Table 3 displays the N_e estimates obtained by the R Package NB for the French populations, after linear extrapolation of Ne estimates and CIs to correct for generational distances among sampled years. For Tourbière de Marchiennes, an estimate of 39.5 was calculated, but with an upper CI equal to the maximal N_e prior of 10000, indicating a great uncertainty associated with the obtained estimate. For Romaine, the estimate was equal to the upper prior of 10000 with

a confidence interval between 0 and 10000. This indicates that the difference in genetic composition caused by sampling is far greater than the genetic drift among sampled intervals, and that no reliable estimate of N_e could be obtained. Only for Tourbière de Vred and Marais de Balançon could we obtain reasonable estimates, which were all extremely low.

Table 3: Variance N_e estimates (with 95% confidence intervals) obtained by the maximum-likelihood-based temporal method, assuming a generation time of three years.

Population	N_e	CI
Tourbière de Vred	12.4	5.5-64.2
Tourbière de Marchiennes	38.5	0-10000
Marais de Balançon	6.5	0-46.9
Romaine	10000	0-10000

4.1.4.2 Linkage Disequilibrium Ne

Table 4 displays inbreeding N_e (adult samples) or bias-adjusted N_b (egg samples) estimates obtained with LDNe (Waples & Do 2008), using the allele frequency threshold of 2%. Estimates were performed for single years and for samples pooled across years. For samples where only eggs were sampled and N_b was estimated, data were also transformed to estimate N_e on the basis of a life table assuming an age span of six years (6Y) or three years (3Y).

Table 4: N_e or N_b (in italic) estimates using linkage disequilibrium. N_b estimates were also transformed to estimate Ne on the basis of a life-table assuming an age span of six years (6Y) or three years (3Y).

Population	Year	Ne or *Nb*	95CI	Ne (6Y)	Ne (3Y)
Vred	2016	25.7	14.7-61.2		
	2017	14.8	9.0-26.6		
	2018	39.7	21.4-118.7		
	Combined	22.5	17.8-28.7		
Marchiennes	2016	3.0	2.3-5.8		
	2017	6.3	3.1-11.3		
	2018	18.1	10.8-36.6		
	Combined	17.1	13.6-21.7		
Balancon	2016	6.5	2.1-21.1		
	2017	17.4	9.7-28.6		
	2018	5.8	2.8-11.0		
	Combined	18.8	13.4-27.1		
Romaine	2018	24.5	15.2-46.4		
	2019	128.0	43.8-inf		
	Combined	102.8	56.8-302.0		

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Zonhoven	2018	*32.8*	*25.3-44.3*	34.1	41.9
Kikbeek	2018	*37.1*	*30.7-45.5*	37.2	45.7
Klein Schietveld	2017	*256.3*	*188.9-376.9*	257.0	316.0
	2019	*126.8*	*55.9-inf*		

Inbreeding N_e estimates showed low to very low effective sizes for Vred, Marchiennes and Balançon, and for Romaine in 2018. For 2019 Romaine showed a much larger estimate, with an infinite upper confidence limit. This generally results from sampling variance being large relative to genetic drift, which indicates an N_e considerably larger than the sample size. For the combined data of 2018 and 2019 (which reduces the sampling variance), the N_e of Romaine was estimated to be around 100, with smaller 95% confidence limits.

As expected, both Kikbeek and Zonhoven showed rather low Ne (35-45, depending on the assumed generation time), whereas Klein Schietveld showed fairly large Ne estimates of 257 to 316. The sample of 2019, of a similar sample size and sample type (adult frogs) as the French populations, and on a smaller spatial scale (c. 1 ha) shows a N_e estimate of around 125, albeit with an infinite upper confidence limit.

4.1.5 Genetic bottlenecks

All seven populations showed and excess of gene diversity compared to expectations at mutation-drift equilibrium (Table 5), although this signal was the least pronounced in Klein Schietveld (lowest value of DH/SD), and the most pronounced in Vred. Among the French populations, the signal was weakest in Romaine. This indicates that all populations have recently undergone population contractions and/or reductions in connectivity with surrounding populations.

Table 5. Summary of bottleneck tests for all populations. P-values are shown for the Standardized difference test and the Wilcoxon test. DH/SD provides the difference in gene diversity divided by the standard deviation of the simulated results per locus, and provides an indication of the strength of the deviation from mutation-drift equilibrium. Negative values indicate population expansion, positive values indicate a bottleneck.

Pop	SD test	Wilc. Test	DH/SD
Balançon	0.0008	0.0002	0.95
Kikbeek	0.0094	0.0042	0.61
Klein Schietveld	0.0263	0.0290	0.52
Marchiennes	0.0047	0.0012	0.72
Romaine	0.0130	0.0067	0.64
Vred	0.0000	0.0002	1.37
Zonhoven	0.0232	0.0034	0.55

5 DISCUSSION

5.1 GENETIC DIVERSITY

All metrics of genetic diversity were lower in French than in Belgian populations. This could have two reasons. Firstly, populations at the edge of a distribution range typically have lower genetic diversity than populations in the core of the range. This can be a result of serial founder effects (Rowe et al. 2006: natterjack toad), or because ecological circumstances are just less favourable at the range edge, and population sizes have always been smaller, as a result of which the equilibrium genetic diversity is also lower. However, the Belgian populations also represent range edge populations, and ecological circumstances in France seemed historically very adequate with large amounts of habitat available (see introduction). The second option is that the lower levels of genetic diversity are the result of stronger recent (last few decades) population declines in French populations. This seems to be corroborated by bottleneck tests, which suggest that the French populations experienced stronger declines. Also the large pairwise genetic distances among neighbouring French populations support that recent genetic drift is at the origin of the lower diversity, rather than phylogeographic history.

5.2 ESTIMATING EFFECTIVE SIZES

Overall, all French populations showed small to very small effective sizes, which is in line with their low levels of genetic diversity and strong inferred declines. However, there are important caveats here with regards to the N_e estimates: the spatial scale of sampling can have a large impact on N_e estimates, especially when moment-based methods are used and the underlying assumption of panmixia is violated (Neel et al. 2013). Moor frogs typically disperse less than 200 m (Brys et al. 2020), and average dispersal distances are likely half of that. Since we can consider the spatial extent of a genetic neighbourhood (the area in which an individual can be expected to spread its genes through offspring) as a circle with radius twice the dispersal distance (Wright 1946) we can estimate the spatial extent of a genetic neighbourhood as an area of approximately 12.5 ha. When sampling discrete areas within a continuous population of a spatial distribution encompassing the area of multiple neighbourhoods, samples restricted to a single neighbourhood area typically reflect the effective neighbourhood size rather than the effective size of the entire population (Neel et al. 2013).

In this study, samples of adult frogs were all taken within an area of a single genetic neighbourhood area: 1 ha for Klein Schietveld, 6 ha for Romaine, 9.5 ha for Balançon, 3.2 ha for Marchiennes and 0.9 ha for Vred. Also egg samples from Kikbeek and Zonhoven came from a single genetic neighbourhood.

In Vred and Marchiennes, the entire known area of occurrence was sampled, and we can hence consider our estimates to represent the total N_e. This isn't the case, however, for the other populations.

In Romaine, moor frogs are currently only known from an area of 12 ha, and we may have received samples from the entire area of occurrence. The vegetation maps and aerial photographs, however, suggest that more habitat might be available and maybe even occupied. Future investigations in the distribution are needed to establish this. The difference in N_e estimates among years (not observed to that scale among other French populations) was likely caused by a larger N_e in 2019. Close monitoring of this population is warranted.

In Balançon the population is thought to occur over a total area of at least 50 ha. Assuming the densities are constant across this area and the same neighbourhood was sampled each consecutive year, the total N_e might be four times larger (N_e approaching 80) than our estimates from a single genetic neighbourhood. Nevertheless, the stark difference with the Klein Schietveld population is striking, where a similar adult sample size (N=31) of a single neighbourhood (1 ha) yielded a sixfold N_e estimate.

For Klein Schietveld we have more accurate estimates of the total N_e, based on a larger sample of eggs, from an area of occurrence of at least 110 ha. Total N_e in our sample of 172 eggs was estimated to be around 320 (assuming a 3 year life span). Looking at the spatial distribution of moor frogs in Klein Schietveld, there are around four more or less discrete neighbourhoods (Fig. 17 in Brys et al. 2020), which could explain the observed discrepancy. Brys et al. (2020) estimated the N_b on the basis of an even larger sample (c. 770 genotypes) to be around 401, or 509 when the four neighbourhoods are taken into account (K. Cox, unpublished results).

The entire Zonhoven population is distributed over c. 90 ha, with six main ponds where reproduction occurs. We only sampled the site with the highest density of moor frogs. It is well possible that our estimate of N_e=42 represents a three- to sixfold underestimation of the true effective population size. Likewise, the Kikbeek population is distributed over 56 ha, with at least three main reproduction sites. Our estimate of N_e=46 may also represent a two- to threefold underestimation of the total N_e.

Notwithstanding the uncertainty in the effective size of the French populations, the combined results of N_e and genetic diversity parameters indicate that the situation in three of four French populations is very unfavourable: the low effective sizes are likely to reduce fitness every generation considerably (Ne<100,Frankham et al. 2014). With already depleted levels of genetic diversity, this effect is likely even stronger. When N_e falls below 50, it indicates critical genetic erosion occurring at a fast pace (Frankham et al. 2002; Hoarau et al. 2005).

The population of Klein Schietveld, which we know is a large and stable population, seems to show a favourable conservation status for effective size as deduced from the Ne95 value of 295. The N_e estimate and confidence limits for this population fall within the Ne95 value. At least in this population, the observed effective size matches relatively well the potential effective size deduced from the habitat availability of c. 110 ha of marsh land, which would translate into a N_e of 216, assuming a density of 20 adults/ha. Densities of adult moor frogs strongly depend on habitat quality (Drobenkov et al. 2005) and can vary strongly from year to year. Habitat quality should therefore also be considered, not simply its size.

The samples of Kikbeek and Zonhoven also showed low N_e values. Since the sampling extent and type (eggs from independent clutches from a single pond or fen) was smaller than the actual population distribution, our N_e values likely underestimate the true effective size, and only estimated the size of the local neighbourhood. In both areas, moor frogs occur over a considerably larger area. Possibly, the true effective size in these metapopulations is two to ten times larger than what we estimated from a single water body. However, these individual sites are not necessarily functionally connected to each other at the present day. Analyses on more sampling sites will be required for a full appraisal of the genetic conservation status of these two populations.

5.3 GENETIC BOTTLENECKS

All populations seem to have suffered recent declines in their effective size. The bottleneck signal was least pronounced in the largest population (Klein Schietveld), and most pronounced in Vred and Balançon, which are two of the three smallest effective populations. In line with

the genetic diversity summary statistics, all French populations had experienced stronger declines than Belgian populations. Among the French populations, the population with the largest effective size (Romaine) had experienced the least pronounced bottleneck.

Genetic diversity in the Romaine population, which was discovered in 2014 but which seems to have a larger effective size, is about as low as in any of the three other French populations. Small population sizes, the secretive life style of the moor frog, inaccessibility of the sites where the species might be present and confusion with the common frog have likely contributed to its late discovery. This population, however, showed a moderate N_e estimate for 2019, which is good news, but which also requires close monitoring.

5.4 GENETIC DIFFERENTIATION AND CONNECTIVITY AMONG SITES

The lower self-assignment success for Marchiennes, with some individuals being assigned to Vred but not vice-versa, may be the result of (historical) asymmetric gene flow. The single individual being assigned to Zonhoven is more than likely an error due to insufficient resolution of the data. The log likelihood score for this genotype was just marginally larger for Zonhoven than for Marchiennes, and this individual was not identified as a first-generation migrant either. The slightly larger proportion of shared ancestry in Belgian populations (also clear from the PCoA and DAPC ordinations) may be the result of larger population sizes, as a result of which genetic drift is slower to result in clearly distinct clusters.

Among the French populations, only the populations of Tourbière de Vred and Tourbière de Marchiennes might still experience genetic exchange. The combined analyses indicate that contemporary gene flow might still be happening between these two populations. The direction of the main gene flow that was inferred, however, differed between methods. Given that the population in Vred is larger and more habitat is available, gene flow from Vred to Marchiennes seems more likely. Even so, the degree to which gene flow might currently be occurring is likely not enough to ensure functional connectivity: gene flow estimates show current exchange to be much lower than one migrant per generation (0.12 to 0.54 migrants per generation). The best strategy is likely to increase local population sizes so as to increase the absolute number of migrants. Alternatively, assisted exchange between sites might temporarily increase gene flow and allow the populations to behave genetically as a metapopulation, thereby slowing down the rate of genetic drift (Ralls et al. 2020).

5.5 MANAGEMENT AND RESEARCH RECOMMENDATIONS

In order to manage the moor frog populations adequately, genetic information can be crucial to make informed decisions on enlarging populations, increasing the connectivity among populations and allowing assisted gene flow or genetic rescue to increase the fitness of populations that suffered severe genetic bottlenecks. Here we provide recommendations for future research and for management.

5.5.1 Assessing the distribution and habitat occupation

Recently, Brys et al. (2020) developed a very sensitive protocol to detect moor frog in water bodies on the basis of environmental DNA. This detection method uses water samples, preferably taken during the period of reproduction, and uses a species-specific DNA-probe to detect moor frogs with a droplet digital PCR method. Their method might be very useful to get a better view on the habitat occupation in the various French sites, especially those situated in the coastal region.

5.5.2 Assessing population sizes

When evaluating Brys et al. (2020), we can conclude that counting egg clumps is a decent surrogate for N_e-estimations with genetic markers, provided that egg clumps are distinguished genetically from common frog (*Rana temporaria*) spawn. Using the data from their detailed study, we find that 63% of the genetically calculated N_e (LD) was predicted by the number of egg clumps (linear regression r^2=0.626, p=0.00000458). This means that when egg clumps are readily identified and observed, counting these could provide important clues about the effective size of the population in each year, without requiring genotyping. In order to distinguish clutches from common frogs, a genetic sampling is advisable, nevertheless.

5.5.3 Genetic methods and sample sizes

Counting egg clumps may be extremely difficult, however, especially when the extent of the distribution itself is poorly known. In such cases, it might be more convenient to directly use genetic methods. Of the two methods used to estimate effective sizes, the temporal method only performed well in one population (Vred), and yielded marginally useful results in another (Marais De Balançon). These estimates, however, were very similar to the LD-based estimates. The LD-based estimates did not clearly differ among years, except for Romaine. In general, LD-based methods perform well when the true effective size of the populations is small. The larger the N_e becomes, the larger sample sizes are required to achieve reliable estimates. In this case, it seems sample sizes of 30 per sampling year were adequate in most cases, but for larger populations (Klein Schietveld), this was clearly not enough: the upper confidence interval was infinite. Such large confidence intervals are typical when sample sizes are too small, which occurs more easily in large effective populations. Also the Romaine population might require larger sample sizes in the future. For future analyses, we advise to sample at least 50-100 individuals, and to record for each sample the sampling site in detail so as to allow a spatial analysis of genetic structure, and to allow a better insight into the extent of genetic neighbourhoods.

Whether to choose adults or eggs for genetic monitoring is not easy to determine. Adults have the advantage of having a spatially broader genetic neighbourhood and of directly allowing estimating the N_e. Eggs or larvae have the advantage of easier sampling (despite the need to distinguish with common frog spawn), and N_b estimates (of individual cohorts) are more sensitive to annual fluctuations, which might be interesting for monitoring purposes (Luikart et al. in press).

5.5.4 Reaching a favourable conservation status?

To reach a favourable conservation status for genetic criteria, we would need to consider at least three different metapopulations of size Ne95 of 295 or larger in order to maintain the stand-still (Balançon, Romaine, Vred-Marchiennes) and reach the goals of having a total population exceeding N_e=1000.

The only two populations that might still experience gene flow are the populations from Parc Naturel Régional Scarpe-Escaut, situated in Tourbière de Vred and Tourbière de Marchiennes. Our best estimates indicate highly asymmetric gene flow, and below the threshold of one migrant per generation. Since gene flow is the product of the migration rate (which is in itself a function of the distance and matrix permeability between sites and the dispersal capacity of a species) and the effective population size N_e, increasing population sizes through targeted population management can aid populations to reach the required gene flow threshold.

Assuming good ecological conditions and a density of 20 adults/ha, the habitat area required to reach the Ne95 goals per metapopulation is circa 150 ha. Current estimates of the amount of habitat in Vred and Marchiennes are at least ten times smaller. The protected area in these sites sums up to 121 ha, of which 10% is actually considered to be good moor frog habitat. According to our calculations, this would result in a carrying N_e capacity of c. 25, which is not far off from our current estimates (22 for Vred, 17 for Marchiennes). In order to reach the Ne95 goals, the amount of moor frog habitat within these sites would need to increase dramatically, or the quality would have to reach exquisite levels typically only found in the core of the distribution range of moor frogs (Drobenkov et al. 2005).

The Romaine population is clearly poorly known, and the area it occupies might be larger than currently assumed, as the N_e we estimated (at least for 2019 and for the combined 2018-2019 data) was clearly larger than in other French populations. It would be interesting to probe this site in more detail with eDNA (see above) and to monitor this population closely for the following years with genetic methods.

Similarly, the Balançon population is situated in a site that might harbour much more moor frog habitat than what is currently known. The N_e-estimates there indicated a very small size, much smaller than what we would expect from the amount of habitat, but in line with observations of this species in the area. This might be due to either sampling only a small genetic neighbourhood, or because the population is truly that small. Since this population is also genetically the most impoverished and showed the strongest genetic bottleneck signal, it is likely that the small N_e is not merely a sampling artefact, and the population is truly small.

5.5.5 Assisted gene flow?

Assisted gene flow is the practice of artificially adding individuals from another population, in order to reduce inbreeding and increase genetic diversity (Ralls et al. 2018). Inbreeding typically leads to inbreeding depression, and even mild inbreeding is known to reduce fitness considerably (Frankham et al. 2014). Even though we didn't investigate inbreeding depression, there is ample evidence in the literature that inbreeding depression is a real issue in small populations. Moreover mimicking natural levels of gene flow (in the order of 1 – 5 migrants per generation) among closely related populations is unlikely to harm populations, and might give them a boost by masking recessive deleterious alleles (Madsen et al. 1999; Ralls et al. 2020 Ebert et al. 2002). Even though the population studied here are genetically clearly differentiated, the proximal reason for this differentiation is the loss of genetic diversity by chance events (genetic drift), rather than natural selection. Countering this process by allowing low to moderate levels of assisted gene flow is expected to have a net positive effect in the short and long term (Madsen et al. 2004; Ralls et al. 2018; Ralls et al. 2020).

Given the much lower levels of genetic diversity in French populations, we would advise genetic exchange through assisted gene flow among all four populations, thereby increasing gradually genetic diversity over time (Ralls et al. 2020). Long-term success, however, will depend ultimately on the availability of sufficient habitat, which is currently a bottleneck for Tourbière de Vred and Tourbière de Marchiennes. The effect of assisted gene flow could be monitored closely at the same time as genetic monitoring of the effective size of the populations takes place.

References

Alterra. (2001). Handboek Robuuste Verbindingen; ecologische randvoorwaarden. Wageningen: Alterra, Research Instituut voor de Groene Ruimte.

Bigot B, Caron A, Holliday J, Janezak A & Fourmy F. (2013). Document d'objectifs NATURA 2000. Diagnostics, enjeux, objectifs et mesures de gestion: Marais de Balançon. Site FR 3110083.

Blondel B (2014) Redécouverte de la grenouille des champs (Rana arvalis) en Picardie (Ponthoile, 80). Avocette 38: 26-28.

Broquet T, Angelone S, Jaquiery J, Joly P, Lena J-P, Lengagne T, Plenet S, Luquet E & Perrin N (2010) Genetic bottlenecks driven by population disconnection. Conservation Biology 24: 1596-1605. 10.1111/j.1523-1739.2010.01556.x

Brys R, Cox K & Neyrinck S. (2020). Ontwikkeling van een monitoringsprotocol voor heikikker: bepaling van de aanwezigheid en verhouding bruine en heikikker via (e)DNA-barcoding en opstart genetische monitoring. Brussel. doi.org/10.21436/inbor.17686189

Colazza S & Bauwens D. (2003). Aanwijzen van prioritaire soorten voor het natuurbeleid in de provincie Limburg. (Verslag van het Instituut voor Natuurbehoud; No. 5). Instituut voor Natuurbehoud.

Cornuet JM & Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144: 2001-2014.

De Saeger S, Wackenier M & Denys L (2018) PAS-gebiedsanalyse in het kader van herstelmaatregelen voor BE2100016 Klein en Groot Schietveld. Rapporten van het Instituut voor Natuur- en Bosonderzoek.

Drobenkov SM, Novitsky RV, Kosova LV, Ryzhevich KK & Pikulik MM (2005) The Amphibians of Belarus. Advances In Amphibian Research In The Former Sovjet Union 10. Pensoft Publishers, Sofia - Moscow. p. 177.

Dufour & Triplet. (2009). Syndicat Mixte Baie de Somme Grand Littoral Picard: Document d'objectifs des marais arrière-littoraux picards. 95 pages + annexes

Ebert D, Haag C, Kirkpatrick M, Riek M, Hottinger JW & Pajunen VI (2002) A selective advantage to immigrant genes in a Daphnia metapopulation. Science 295: 485-488.

Fog K & Hesselsøe M (2009) Udvikling af prototypemodel til brug for forvaltning af spidssnudet frø i forbindelse med vejanlæg. Amphi Consult.

Frankham R (1995) Effective population size/adult population size ratios in wildlife: a review. Genetical Research 66: 95-107.

Frankham R (2005) Genetics and extinction. Biological Conservation 126: 131-140.

Frankham R, Balloux JD & Briscoe DA. (2002). Introduction to Conservation Genetics: Cambridge University Press. 617 p.

Frankham R, Bradshaw CJA & Brook BW (2014) Genetics in conservation management: Revised recommendations for the 50/500 rules, Red List criteria and population viability analyses. Biological Conservation 170: 56-63. 10.1016/j.biocon.2013.12.036

Goudet J (2005) Hierfstat, a package for R to compute and test hierarchical F-statistics Molecular Ecology Notes 5: 184-186.

Gyovai F (1989) Demographic analysis of the moor frog (Rana arvalis Wolterstorffi Fejérváry 1919) population in Fraxino pannonicae - Alnetum of the Tisza basin. Tiscia 24: 107-122.

Hamilton WD. (2009). Population Genetics. Chichester UK: Wiley-Blackwell.

Hoarau G, Boon E, Jongma DN, Ferber S, Palsson J, Van der Veer HW, Rijnsdorp AD, Stam WT & Olsen JL (2005) Low effective population size and evidence for inbreeding in an overexploited flatfish, plaice (Pleuronectes platessa L.). Proceedings of the Royal Society B-Biological Sciences 272: 497-503. 10.1098/rspb.2004.2963

Houlahan JE, Findlay CS, Schmidt BR, Meyer AH & Kuzmin SL (2000) Quantitative evidence for global amphibian population declines. Nature 404: 752-755. 10.1038/35008052
Hui T-YJ & Burt A (2015) Estimating effective population size from temporally spaced samples with a novel, efficient maximum-likelihood algorithm. Genetics 200: 285-293. 10.1534/genetics.115.174904
Jaeger JAG, Soukup T, Madriñán LF, Schwick C & Kienast F. (2011). Landscape fragmentation in Europe. Joint EEA-FOEN report. Copenhagen, Denmark: EEA.
Jombart T (2008) adegenet: a R package for the multivariate analysis of genetic markers. Bioinformatics 24: 1403-1405. 10.1093/bioinformatics/btn129
Jombart T & Collins C. (2015). A tutorial for Discriminant Analysis of Principal Components (DAPC) using adegenet 2.0.0.
Jooris R, Engelen P, Speybroeck J, Lewylle I, Louette G, Bauwens D & Maes D. (2013). De amfibieën en reptielen van Vlaanderen. Recente verspreiding en toelichting bij de nieuwe Rode Lijst. Rapport Natuurpunt.Studie 2013/6, Mechelen.
Kuzmin S, Tarkhnishvili D, Ishchenko V, Tuniyev B, Beebee T, Anthony BP, Schmidt B, Ogrodowczyk A, Ogielska M, Babik W, Vogrin M, Loman J, Cogalniceanu D, Kovács T & Kiss I. (2009). Rana arvalis. The IUCN Red List of Threatened Species 2009: e.T58548A86232114.
Loman J (1984) Density and survival of Rana arvalis and Rana temporaria. Alytes 3: 125-134.
Luikart G, Antao T, Hand BK, Muhlfeld CC, Boyer MC, Cosart T, Trethewey B, Al-Chockhachy R & Waples RS (in press) Detecting population declines via monitoring the effective number of breeders (Nb). Molecular Ecology Resources n/a. 10.1111/1755-0998.13251
Madsen T, Shine R, Olsson M & Wittzell H (1999) Restoration of an inbred adder population. Nature 402: 34-35.
Madsen T, Ujvari B & Olsson M (2004) Novel genes continue to enhance population growth in adders (Vipera berus). Biological Conservation 120: 145-147. 10.1016/j.biocon.2004.01.022
Mergeay J. (2013). Analyse van de mogelijke verbindingen voor amfibieën en reptielen in de S-IHD rapporten. Adviezen van het Instituut voor Natuur- en Bosonderzoek. INBO.A.2013.66.
Mergeay J, De Blust G, Vandekerkhove K, Thomaes A & De Becker P. (2018). PAS-GEBIEDSANALYSE in het kader van herstelmaatregelen voor BE2200031 Valleien van de Laambeek, Zonderikbeek, Slangebeek en Roosterbeek met vijvergebieden en heiden. Rapporten van het Instituut voor Natuur- en Bosonderzoek 2018 (37). Instituut voor Natuur- en Bosonderzoek, Brussel. doi.org/10.21436/inbor.14323209
Mergeay J & Van Hove M. (2013). Analyse van de duurzaamheid van populaties van Europees beschermde amfibieën en reptielen (deel 2). Adviezen van het Instituut voor Natuur- en Bosonderzoek. INBO.A.2013.104.
Mills LS & Allendorf FW (1996) The one-migrant-per-generation rule in conservation and management. Conservation Biology 10: 1509-1518.
Neel MC, McKelvey K, Ryman N, Lloyd MW, Short Bull R, Allendorf FW, Schwartz MK & Waples RS (2013) Estimation of effective population size in continuously distributed populations: there goes the neighborhood. Heredity 111: 189-199. 10.1038/hdy.2013.37
Nöllert A & Nöllert C. (2001). Amfibieëngids van Europa. Baarn, Nederland: Tirion Uitgevers BV.
Paelinckx D. (2009). Gewestelijke doelstellingen voor de habitats en soorten van de Europese Habitat- en Vogelrichtlijn voor Vlaanderen. Mededelingen van het Instituut voor Natuur- en Bosonderzoek INBO.M.2009.6, Brussel, 669 p.
Paetkau D, Slade R, Burden M & Estoup A (2004) Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation-based exploration of accuracy and power. Molecular Ecology 13: 55-65. 10.1046/j.1365-294x.2004.02008.x

//

Palo JU & Merilä J (2003) A simple RFLP method for identification of two ranid frogs. Conservation Genetics 4: 801-803. 10.1023/B:COGE.0000006117.43571.6e

Palstra FP & Ruzzante DE (2008) Genetic estimates of contemporary effective population size: what can they tell us about the importance of genetic stochasticity for wild population persistence? Molecular Ecology 17: 3428-3447. 10.1111/j.1365-294x.2008.03842.x

Piry S, Alapetite A, Cornuet J-M, Paetkau D, Baudouin L & Estoup A (2004) GeneClass2: A software for genetic assignment and first-generation migrant detection. Journal of Heredity 95: 536-539.

Pontoppidan M & Nachmann G (2013) Spatial Amphibian Impact Assessment - A management tool for assessment of road effects on regional populations of Moor frogs (Rana arvalis). Nature Conservation 5: 29-52.

R_Core_Team. (2018). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ralls K, Ballou JD, Dudash MR, Eldridge MDB, Fenster CB, Lacy RC, Sunnucks P & Frankham R (2018) Call for a paradigm shift in the genetic management of fragmented populations. Conservation Letters 11: e12412. 10.1111/conl.12412

Ralls K, Sunnucks P, Lacy RC & Frankham R (2020) Genetic rescue: A critique of the evidence supports maximizing genetic diversity rather than minimizing the introduction of putatively harmful genetic variation. Biological Conservation 251: 108784. 10.1016/j.biocon.2020.108784

Rannala B & Mountain JL (1997) Detecting Immigration by Using Multilocus Genotypes. Proceedings of the National Academy of Sciences of the United States of America 94: 9197-9201.

Rondel S, Duhayon G, Godin J & Lemoine G (2013) Bilan de trois années de suivi des effectifs reproducteurs et des habitats de reproduction de la Grenouille des champs dans la plaine de la Scarpe (Nord), perspectives de préservation. Revue Scientifique de Bourgogne - Nature 17: 180-198.

Rousset F (2008) Genepop'007: a complete reimplementation of the Genepop software for Windows and Linux. Molecular Ecology Resources 6: 103-106.

Rowe G, Harris DJ & Beebee TJC (2006) Lusitania revisited: A phylogeographic analysis of the natterjack toad Bufo calamita across its entire biogeographical range. Molecular Phylogenetics and Evolution 39: 335-346.

RStudio_Team. (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/.

Severtsov AS, Liapkov SM & Surova GS (1998) The correlation of the ecological niches of the common (Rana temporaria L.) and of the moor (Rana arvalis Nilss.) frogs (Anura, Amphibia). Zh Obshch Biol 59: 279-301.

Smith AM & Green DM (2005) Dispersal and the metapopulation paradigm in amphibian ecology and conservation: are all amphibian populations metapopulations? Ecography 28: 110-128. 10.1111/j.0906-7590.2005.04042.x

Speybroeck J & De Knijf G. (2019). Staat van instandhouding (status en trends) van de soorten van de Habitatrichtlijn. Deelrapport amfibieën en reptielen - rapportageperiode 2013-2018. Rapporten van het Instituut voor Natuur- en Bosonderzoek. Instituut voor Natuur- en Bosonderzoek, Brussel. doi.org/10.21436/inbor.16089660

UICN-France, MNHN & SHF. (2015). La Liste rouge des espèces menacées en France - Chapitre Reptiles et Amphibiens de France métropolitaine. Paris, France.

Vacher J-P (2010) Statut actuel de la grenouille des champs Rana arvalis Nilsson, 1842 (Amphibia: Ranidae) sur la bande rhénane en Alsace. Bulletin de La Société d'Histoire Naturelle et d'Ethnographie de Colmar 69: 3-12.

Van Oosterhout C, Hutchinson WF, Wills DPM & SHIPLEY P (2004) micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Molecular Ecology Notes 4: 535-538. 10.1111/j.1471-8286.2004.00684.x

Wang J, Santiago E & Caballero A (2016) Prediction and estimation of effective population size. Heredity 117: 193-206. 10.1038/hdy.2016.43

Waples RS, Antao T & Luikart G (2014) Effects of Overlapping Generations on Linkage Disequilibrium Estimates of Effective Population Size. Genetics. 10.1534/genetics.114.164822

Waples RS & Do C (2008) LDNE: a program for estimating effective population size from data on linkage disequilibrium. Molecular Ecology Resources 8: 753-756. 10.1111/j.1755-0998.2007.02061.x

Waples RS & Do C (2010) Linkage disequilibrium estimates of contemporary N-e using highly variable genetic markers: a largely untapped resource for applied conservation and evolution. Evolutionary Applications 3: 244-262. 10.1111/j.1752-4571.2009.00104.x

Wilson GA & Rannala B (1997) Bayesian inference of recent migration rates using multilocus genotypes. Genetics 163: 1177-1191.

Wright S (1946) Isolation by distance under diverse systems of mating. Genetics 31: 39-59.

Appendices

Table A1. *R. arvalis* conservation status assessment per member state per biogeographical region for the period of 2007-2012 as reported according to article 17 of the Habitats Directive. ALP: alpine region. ATL: atlantic region. BOR: boreal region. CONT: continental region. PAN: Pannonian region. The result of the assessment for each parameter of conservation status are presented in four categories: 'favourable' (FV), 'unfavourable-inadequate' (U1), 'unfavourable-bad' (U2) and 'unknown' (XX). Signs (-, +, =) further modify the overall appraisal.

Member state	Region	Range	Population	Habitat	Overall
Austria	ALP	U1-	U1	U1	U1=
Poland	ALP	XX	XX	XX	XX
Romania	ALP	U1=	U1=	U1=	U1-
Sweden	ALP	FV=	FV=	FV=	FV
Slovenia	ALP	XX	XX	U1-	U1-
Belgium	ATL	FV+	FV=	U1=	U1+
Germany	ATL	U1-	U1-	U1-	U1-
Denmark	ATL	FV	FV	FV	FV
France	ATL	U1=	FV=	FV=	U1=
The Netherlands	ATL	FV=	FV=	FV=	FV
Estonia	BOR	FV+	FV+	FV=	FV
Finland	BOR	FV=	FV	FV=	FV
Lithuania	BOR	FV=	U1+	U1	U1=
Latvia	BOR	FV	FV	FV+	FV
Sweden	BOR	FV=	FV=	FV=	FV
Austria	CON	U1=	U1	U1-	U1-
Czech Republic	CON	FV=	U1-	U1-	U1-
Germany	CON	U1-	U1-	U1-	U1-
Denmark	CON	U1	U1	U1	U1
France	CON	U2-	U2	U1=	U2-
Poland	CON	FV=	FV	FV	U1
Romania	CON	U1=	U1=	U1-	U1-
Sweden	CON	FV=	FV=	FV	FV
Slovenia	CON	XX	XX	U1-	U1-
Czech Republic	PAN	FV=	FV=	U1-	U1-
Hungary	PAN	U1=	U1=	U1=	U1=

Romania	PAN	U1-	U1	U1-	U1-
Slovakia	PAN	U2-	U1-	U2=	U2=
Total proportion inadequate		0.39	0.46	0.61	0.68

Table A2: Natura2000 habitat codes of *R. arvalis*' typical terrestrial and aquatic habitat types in France and Belgium

Habitat code	Habitat code
2310	Psammophile heather with *Calluna* and *Genista* species
4010	North Atlantic moist heather with *Erica tetralix*
4030	Dry European heather
6230	Species-rich Nardus grasslands
6410	Molinia meadows on calcareous, peaty or clayey-silt-laden soils
7110	Active peat bog
7140	Transition mires and quaking bog
7150	Depressions on peat substrates (Rhynchosporion)
7230	Alkaline fens
3110	Oligotrophic waters containing very few minerals of sandy plains (Littorelletalia uniflorae)
3130	Oligotrophic to mesotrophic standing waters with vegetation of the Littorelletea uniflorae and/or of the Isoeto-Nanojuncetea
3160	Dystrophic natural pools and lakes

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Table A3. Overview of samples per year and site. Actual number of genotypes can be slightly smaller, as some DNA samples were of too low quality.

Year	Locality	Sample type	# samples
Belgium			
2017	Klein Schietveld	Eggs	172
2018	Kikbeek	Eggs	60
	Zonhoven	Eggs	42
2019	Klein Schietveld	Adults	31
France			
2016	Marais de Balançon	Adults	10
	Tourbière de Vred	Adults	25
	Tourbière de Marchiennes	Adults	14
2017	Marais de Balançon	Adults	38
	Tourbière de Vred	Adults	30
	Tourbière de Marchiennes	Adults	14
2018	Marais de Balançon	Adults	26
	Tourbière de Vred	Adults	30
	Tourbière de Marchiennes	Adults	19
	Marais de Romaine	Adults	30
2019	Marais de Romaine	Adults	34

Table A4. List of microsatellite markers tested. Only markers in bold were retained for this study. MP indicates in which multiplex mix each marker was used. Fl: fluorescent. PCR reactions were performed with final DNA concentrations of 5 ng/µl, in a final volume of 10 µl containing 5 µl of Qiagen Multiplex Master Mix. cycling conditions used annealing temperatures of 50°C (MP1, 35 cycles), 55°C (MP2, 35 cycles). Marker RA13 was used separately in a touch-down PCR for the first 22 cycles (starting at 60°C, decreasing 0.5°C per cycle), followed by 15 cycles at 50°C. Marker RtU4 used 35 cycles at 46°C annealing. PCR conditions started with 15 min at 95°C, followed by cycles of 30s 95°C, 30s annealing and 30s extension at 72°C, with a final terminal elongation at 72°C.

Marker	MP n°	Fl Label	Conc (µM)	Reference / Genbank Accession n°
RCIDII	1	PET	0.2	Vos et al., 2001
RNTYR2	1	PET	0.6	Vos et al., 2001
RA14	1	NED	0.2	EU871714
RtsB14	1	VIC	0.2	Berlin et al., 2000
RlatCa41	1	FAM	0.2	Garner & Tomio, 2001
RECALQ	1	PET	0.2	Vos et al., 2001
RC08604	1	FAM	0.4	Vos et al., 2001
RRD590	2	NED	0.2	Vos et al., 2001
RlatCa18	2	VIC	0.2	Garner & Tomio, 2001
Rtempµ9	2	FAM	0.05	Rowe & Beebee, 2001
RA11	2	PET	0.2	EU871712
Rt2Ca2-22	2	FAM	0.2	T. Garner, unpublished
RtµP	2	NED	0.2	Pidancier et al., 2002
Rtempµ4	2	NED	0.05	Rowe & Beebee, 2001
Rtempµ5	2	PET	0.4	Rowe & Beebee, 2001
RA03	2	FAM	0.4	EU871710
RA04	2	VIC	0.2	EU871711
RA13	3	VIC	0.4	EU871713
RtU4	4	FAM	0.4	Berlin et al., 2000

Table A5. Life table of fecundity and survival rates per age class.

Stage/Age	Survival probability	Fecundity
Egg/larvae	0.005	/
0	0.55	0
1	0.55	70
2	0.55	945
3	0.55	1190
4	0.5	1250
5	0.4	1300

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6	0.2	1300

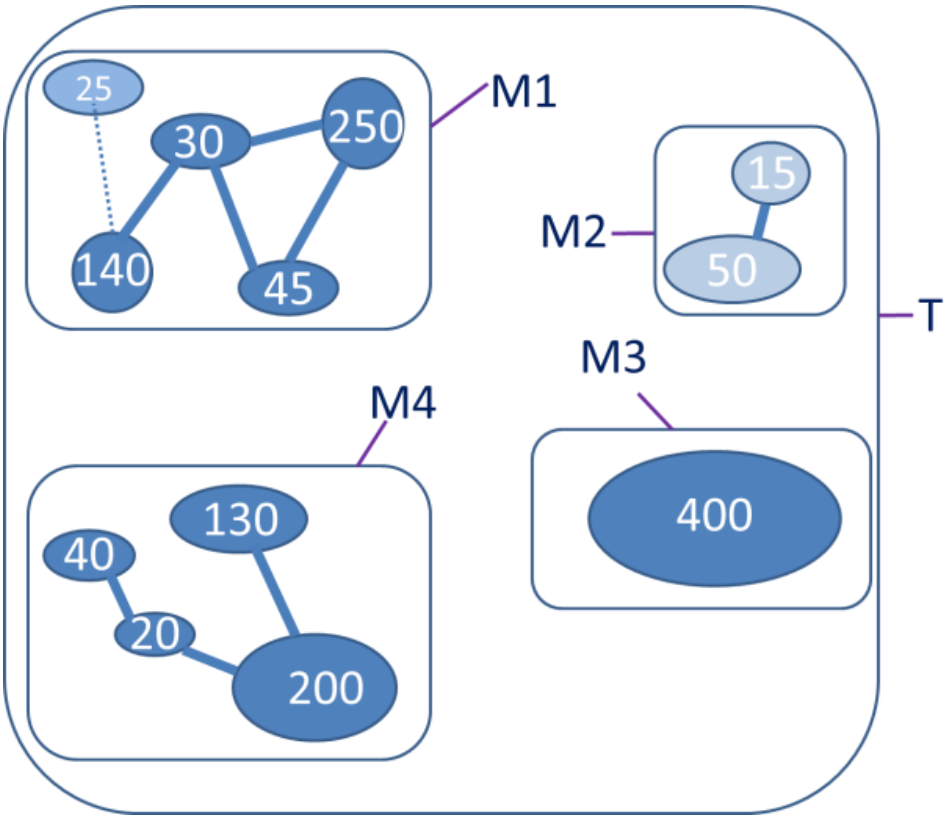

Figure A1: Visual representation of a total population (T) consisting of multiple metapopulations (M) with functionally connected subpopulations. Numbers represent the effective populations size (Ne) of subpopulations. A local population meets the Ne95 criterium if it meets the Ne95 itself or if it is part of a metapopulation that does so. The total population has a FCS if the sum of Ne's of all populations meeting the Ne95 criterium is larger than 1000. Full lines connecting populations represent adequate gene flow (>1 migrant per generation), dotted lines represent insufficient gene flow. In this theoretical example, M1 consists of five subpopulations, of which one is not sufficiently connected. The resulting effective size of M1 =140+250+30+45=465 (not counting the insufficiently connected subpopulation), which exceeds the Ne95 value of 295. We can conclude that M1 has a favourable conservation status. M2 is below the Ne95 threshold. M3 consists of just a single population, which is larger than Ne95. Also M4 is larger than Ne95. To calculate the total effective size, we exclude M2 as it is smaller than Ne95, and sum the other three metapopulations. T=465+390+400=1255, which is larger than 1000. We can conclude that for genetic criteria, this total population has a favourable conservation status.

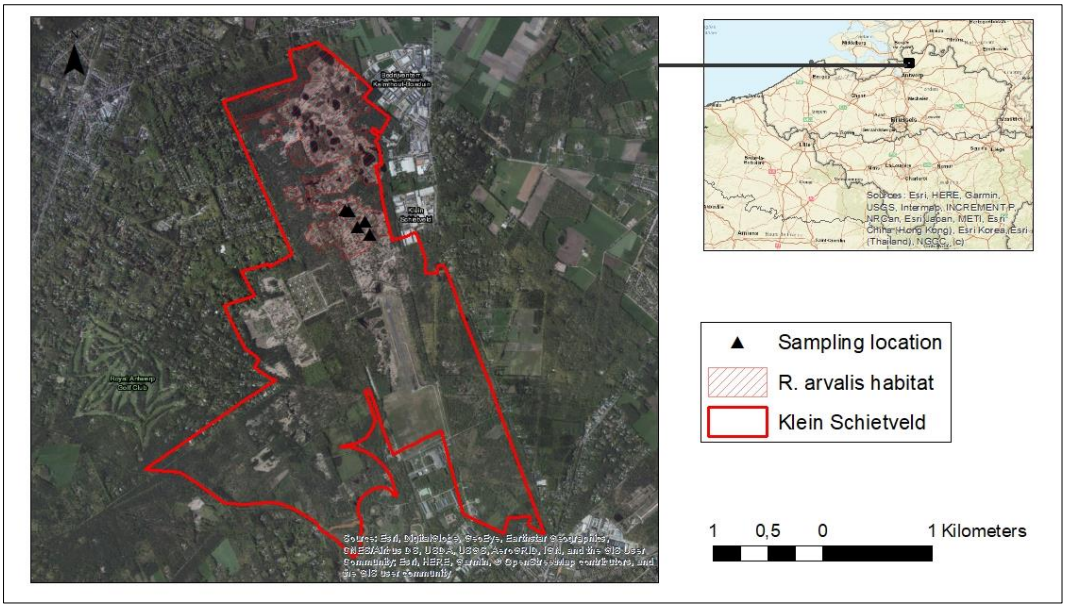

Figure A2. Map of Klein Schietveld, showing the estimated occupied *R. arvalis* habitat and the Adult sampling locations. Eggs were sampled over the entire habitat area

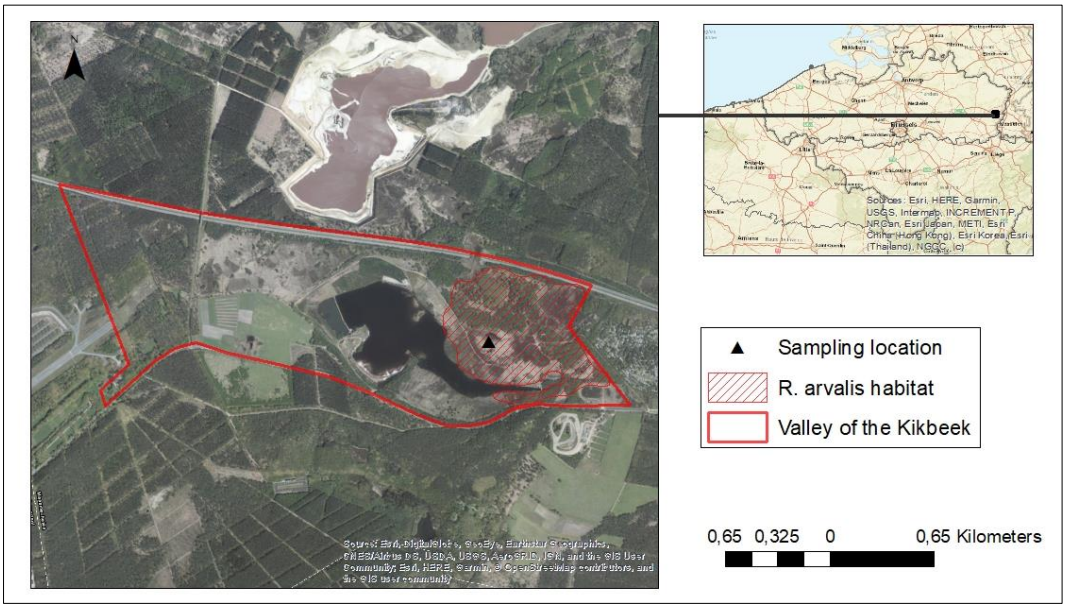

Figure A3. Map of Kikbeek, showing the estimated occupied *R. arvalis* habitat and the eggs sampling location.

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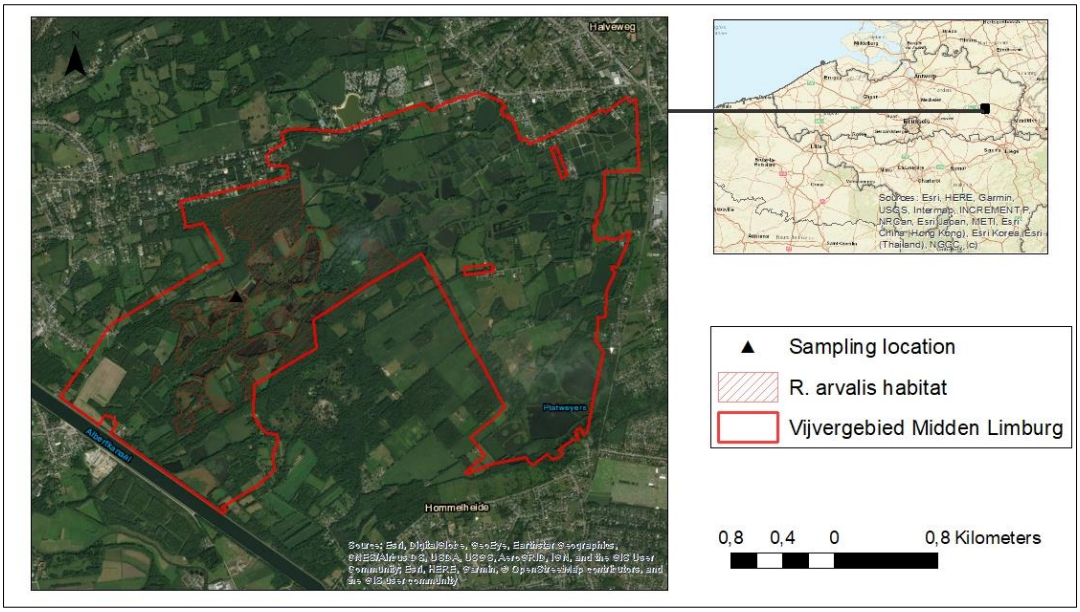

Figure A4. Map of Vijvergebied Midden Limburg, Zonhoven, showing the estimated occupied *R. arvalis* habitat and the eggs sampling location.

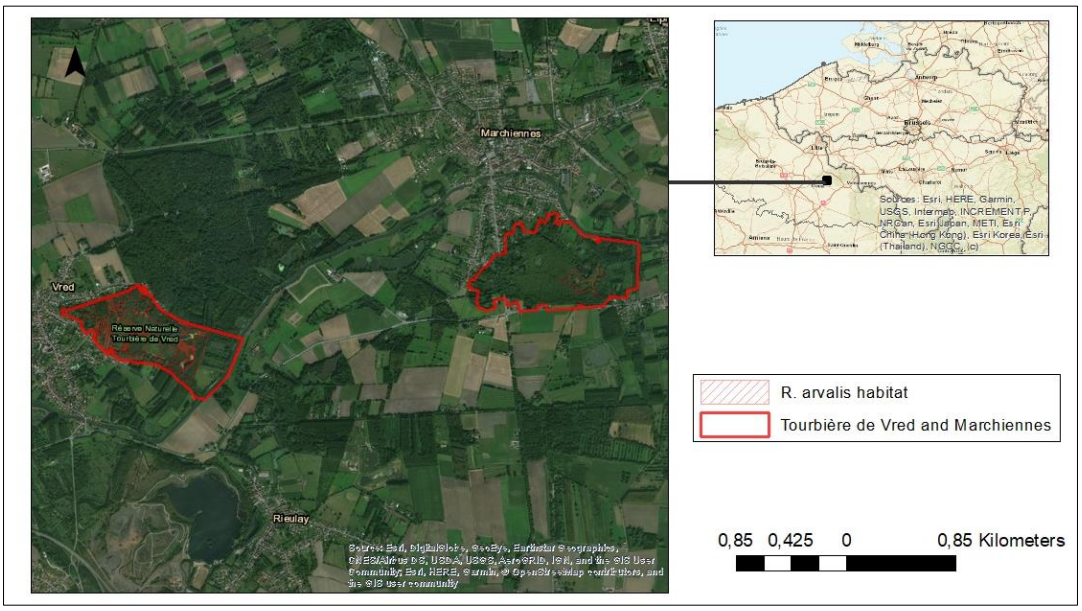

Figure. A5. Map of Tourbière de Vred (left) and Marchiennes (right), showing the estimated occupied *R. arvalis* habitat.

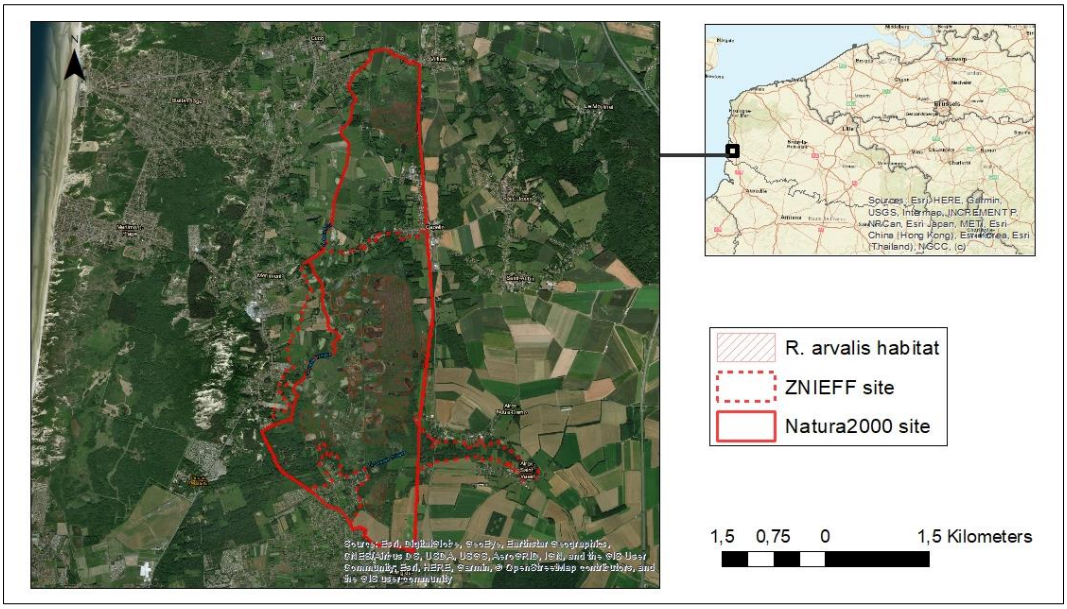

Figure A6. Map of Marais de Balançon Natura2000 and ZNIEFF sites, showing the estimated occupied *R. arvalis* habitat.

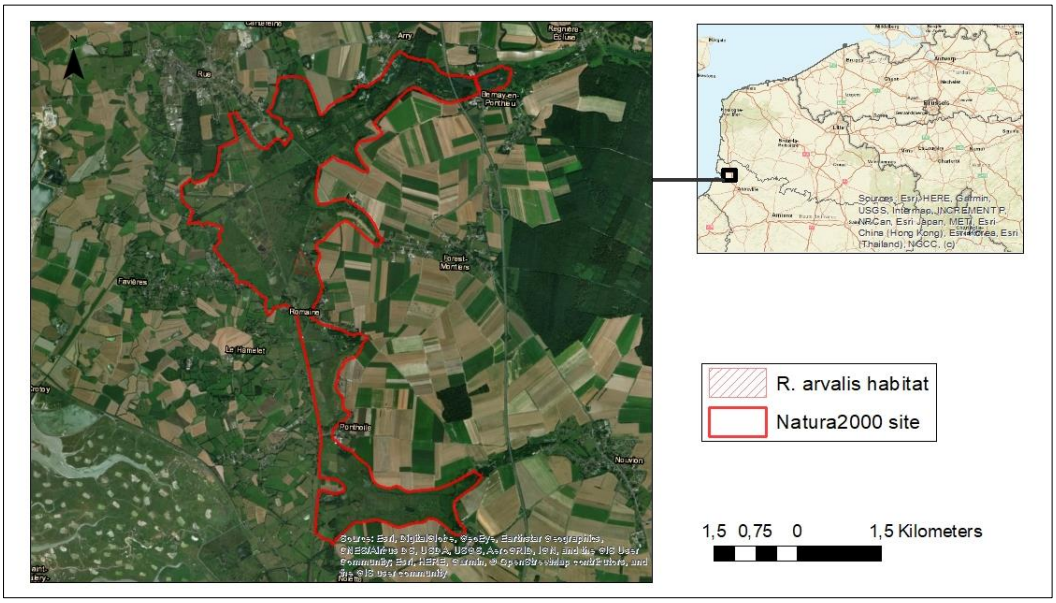

Figure A7. Map of the "Marais arrière-littoraux picards" Natura2000 site, showing the estimated occupied *R. arvalis* habitat at Marais de Romaine.

doi.org/10.21436/inbor. 19362192