

**Phylogeography and Population Genetics of the Moor frog
(*Rana arvalis* Nilsson) in Northern Europe**

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Academic Dissertation

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This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Knopp T, Merilä J (2008) The postglacial recolonization of Northern Europe by *Rana arvalis* as revealed by microsatellite and mitochondrial DNA analyses. *Heredity*, in press.
- II Knopp T, Merilä J (2008) Microsatellite variation and population structure of the moor frog (*Rana arvalis*) in Scandinavia. *Manuscript*.
- III Knopp T, Cano JM, Crochét P-A, Merilä J (2007) Contrasting levels of variation in neutral and quantitative genetic loci on island populations of moor frogs (*Rana arvalis*). *Conservation Genetics*, **8**, 45-56.
- IV Knopp T, Heimovirta M, Kokko H, Merilä J (2008) Do male moor frogs (*Rana arvalis*) lek with kin? *Molecular Ecology*, **17**, 2522-2530.
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INTRODUCTION

Approximately one third of all the amphibian species currently known are threatened by extinction (Stuart et al. 2004). Several extrinsic causes have been suggested for the observed patterns, including increased UV-radiation and pollutants, climate change, diseases, habitat loss and fragmentation (Collins & Storer 2003; Beebe & Griffiths 2005). However, one of the biggest threats to the long-term persistence of amphibian populations, as well as of other species, is the intrinsic loss of genetic variation. In order to adapt to changes in their abiotic and biotic environments, organisms are critically dependent on the existence of heritable variation in traits important for their fitness. In areas where the interpopulation connectivity is reduced and the population sizes are declining, genetic variation will gradually be lost and the risk of extinction will increase (Frankham et al. 2002). Thus, the assessment of genetic diversities, and surveys of the patterns and processes that have shaped the genetic population structures of different species, are central elements in modern conservation practices.

In phylogeographic studies, the intraspecific genetic structuring of populations is interpreted in the light of demographic and historical data (Avice 2000). Phylogeography is intimately related to conservation due to the fact that it allows an identification of management units (MU) and evolutionary significant units (ESU, Moritz 1994). Here, demographically independently acting populations with either shallow (MU) or

deep (ESU) phylogenetic divergence can be identified with genetic methods and managed accordingly. Comparative phylogeographic studies can be employed to recognize areas of particular evolutionary significance, and to identify common biogeographical patterns in regional biotas. In Northern Europe, as well as in Northern America, the broad and shared genetic patterns among terrestrial biota have commonly been interpreted in the light of the Pleistocene glacial events (Hewitt 1996).

Postglacial recolonization of Northern Europe

During the Pleistocene (ca 1.9 Mya-10 Kya) the Eurasian biota has frequently undergone periods of major glaciations. During the cold periods the advancing Arctic ice sheet forced the flora and fauna to seek for refugia in the south (Fig. 1) while the interglacial periods have been characterized by recolonization events. These incidents have evidently had large effects on the speciation, species distribution and within species diversities of both aquatic and terrestrial taxa (Hewitt 1996; Avice 2000).

The last glacial maximum ended around 10 000 years ago. This allowed the terrestrial flora and fauna to expand back to their initial ranges from their main sanctuaries in the peninsula of Iberia, Italy and the Balkans (Fig. 1, Hewitt 1999). The recolonization events were stochastic as demonstrated by the dissimilar phylogeographic patterns among many species.

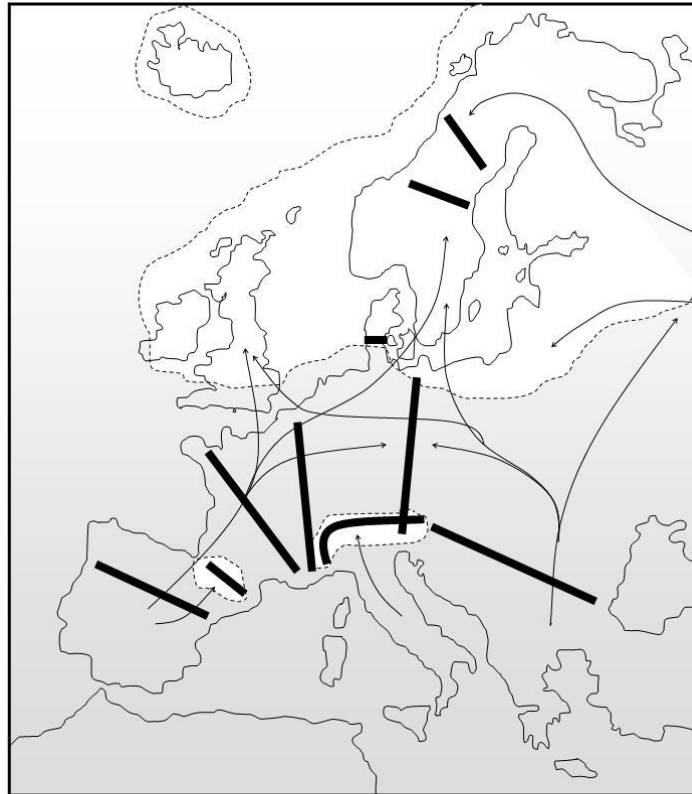


Figure 1. Ice cover (dotted line) in Europe during the last glacial maximum ca. 10 000 years ago. The recolonization routes from the main refugial areas for terrestrial biota are indicated by arrows, the hybrid zones by thick lines (Taberlet et al. 1998; Hewitt 1999).

However, some distinct, repeated patterns have been revealed in the phylogeographic distribution of European biota, particularly among Fennoscandian species. One has been the identification of several common hybrid zones throughout Europe (Fig. 1). Within these zones, the divergent intraspecific genetic lineages stemming from different refugia have met, thus forming areas of increased overall genetic

diversity. In Scandinavia, such an area is located approximately where the final ice sheet melted in northern Sweden. Secondly, the northern populations commonly display reduced levels of within-population genetic diversities when compared to more southern ones. This has been explained as an outcome of the serial bottlenecking that took place during the recolonization process (Hewitt 1996).

Finally, the short time since colonization has resulted in northern populations generally being genetically more homogenous than tropical and temperate zone populations (reviewed in Pamilo & Savolainen 1999). However, the stressful environmental conditions within the boreal zone may temporarily reduce the population sizes and the action of genetic drift will be increased. Consequently, a high degree of genetic differentiation between populations can occur also in the north (Gyllenstein 1985; Mikko & Andersson 1995).

The population genetic structure of amphibians

Perhaps one of the most significant inductions to our understanding of the genetic structuring of amphibian populations was Ward et al.'s (1992) comparative study of allozyme polymorphism in different animal taxa. In that survey, Ward et al. (1992) demonstrated that of all the major classes of animals, the amphibians exhibit the highest degree of population subdivision as measured by allozyme markers. Later on, molecular markers have provided additional insights on amphibian population dynamics, migration and dispersal patterns (Rowe et al. 1998; Palo et al. 2004a,b; Johansson et al. 2005; Cabe et al. 2007). Though some discrepancies among species have been revealed, studies based on microsatellite markers have confirmed that amphibians generally display a high degree of genetic structuring at all scales. A significant genetic differentiation has been discovered in

recently colonized areas and between adjacent populations (Vos et al. 2001; Palo et al. 2004a). Exceptions to this pattern have been detected only in a few studies (Funk et al. 2005) or species in which a metapopulation structure has been suggested (reviewed in Smith & Green 2005). The dependence on both aquatic and terrestrial habitats, as well as the philopatric behaviour, makes amphibians susceptible to anthropogenically induced changes in their environment. Pollution, habitat loss, and dispersal barriers have all been shown to affect amphibian populations negatively (Collins & Storer 2003; Hitchings & Beebe 1998; Vos & Chardon 1998; Andersen et al. 2004) contributing to the fact that more than 40% of amphibian species are currently undergoing population declines (Stuart et al. 2004).

In focus: Rana arvalis

I chose the moor frog, *Rana arvalis* as a study species since it possesses a number of qualities making it a highly interesting model in a population and conservation genetic context. Firstly, *R. arvalis* has declined dramatically in central Europe and it has been included in appendix II of the Bern convention (1979) as well as in annex IV of the species directive of EU (Council directive 92/43/EEC 1992). Hence, there is a formal agreement to protect not only the species itself, but also the habitats it occupies. Consequently, information on factors influencing its population dynamics, including data on its genetic structuring and dispersal, are needed. Secondly, the species show a clear

transition from one colour morph to another across Fennoscandia (Fog et al. 1997). This has evoked a hypothesis of a two-directional recolonization of Fennoscandia since the last glacial maximum (Fog et al. 1997). However, as the two colour morphs may also reflect selective (ecological) rather than phylogenetic (i.e. historical) differentiation, genetic data can be utilized in attempts to differentiate between these two explanations. Thirdly, quantitative

genetic research in this species has revealed a high a degree of genetic differentiation in ecologically important traits even over relatively short geographic distances (Räsänen et al. 2003 a,b). Combining these data with population genetic studies based on neutral genetic markers, it is possible to gain insights into the causes behind the population differentiations seen in quantitative traits (e.g. Leinonen et al. 2008).

Box 1. Facts about the moor frog, *Rana arvalis*

Rana arvalis

- ⦿ 35-75 mm in size
- ⦿ during a short breeding season in early spring, each female lays 400-1600 eggs
- ⦿ reach metamorphosis in 60- 90 days
- ⦿ mature at 2-4 years



A lek of males calling for females



Moor frog males turn blue during the most intensive part of the breeding season

- ⦿ inhabits damp fields, alder swamps, low-land moors and meadows across Eurasia
- ⦿ one of the most common anuran species in Scandinavia
- ⦿ has suffered from habitat loss and fragmentation in many parts
- ⦿ is strictly protected by law

RESEARCH OBJECTIVES

In this thesis, my aim has been to study population differentiation and colonization history of Fennoscandian moor frogs (*Rana arvalis*) from both historical (evolutionary) and applied (conservation) perspectives using molecular markers as a tool.

Genetic population structure, phylogeography and historical demography of *Rana arvalis* in Northern Europe

The rationale behind these studies was to explore the basic genetic population structure of the species across Europe, with a special emphasis on Fennoscandia (**I, II, III**). The main aim was to uncover the broad patterns of population subdivision and find the scale in which significant gene flow can be expected to occur among populations (**II**). Also potential differences in dispersal patterns between the sexes were considered (**II**).

An important part of this research was to test the multiple re-colonization route hypothesis of Fennoscandia. Using both microsatellite markers and mitochondrial DNA sequence data, I aimed to identify possible divergent lineages that have colonized the area, and to see whether the results are congruent with the distribution of the two colour morphs found in *Rana arvalis* (**I**).

One specific question concerned *R. arvalis* on the Baltic island of Gotland (**III**). Phenotypic and quantitative genetic data had indicated strong differentiation between mainland and Gotland populations (Nilson & Andrén 1981, Lardner 1995,

Laurila et al. 2002). It had been suggested that the Gotlandic *R. arvalis* could constitute a sub-species of its own (Fog et al. 1997, Lindgren 2001). By analyzing data from both mainland and two other islands, the degree of genetic differentiation was elucidated in a broad context. Moreover, by comparing the differentiation in quantitative traits (Q_{ST}) with the differentiation in neutral markers (F_{ST}) I aimed to gain insight to the relative roles of natural selection and genetic drift in explaining the smaller size of Gotlandic moor frogs as compared to their mainland conspecifics.

***Rana arvalis* leks – their genetic structuring and evolutionary significance**

The last part of my thesis (**IV, V**) aimed to gain insight to the genetic structuring of amphibian populations in their smallest scale; within leks and broods. Like in lekking birds, males of many amphibians – including *R. arvalis* – assemble on a common mating ground in order to attract females (Wells 1977, Höglund & Alatalo 1995). A lot of attention has been given to understand the evolutionary forces behind lek formation, yet basically nothing is known on this subject in amphibians. Thus, the objective was to raise questions on why amphibians form leks, and how do they choose their leks? As a starting point, the kin-selection hypothesis was tested (**IV**). An additional dimension to this study was to examine the existence of multiple paternity, and consider its evolutionary significance in *R. arvalis* populations. (**V**).

MATERIAL AND METHODS

Here are presented only briefly the material and methods used in the studies constituting this thesis. The detailed descriptions of laboratory and analytical procedures can be found in the original papers.

Samples

For the different studies included, altogether 35 *R. arvalis* populations were sampled during the years 1998-2005 (Fig. 2). An emphasis was placed on the populations in Northern Europe, but for the recolonization study (I) additional samples were obtained from Russia and Central

Europe. Sampling was accomplished by collecting adult individuals (I-IV) or eggs (I-V) during the short breeding season in early spring. For the general population genetic studies (I, II, III) each population was sampled at a single location once. In order to avoid the sampling of siblings, only one individual per clutch of eggs from a single female was taken. Kin selection in *R. arvalis* leks was tested by sampling adult males from two populations (IV). In the paternity analyses (V) the sampling scheme consisted of multiple eggs from each female. Permissions for sampling were obtained from the local environmental authorities.

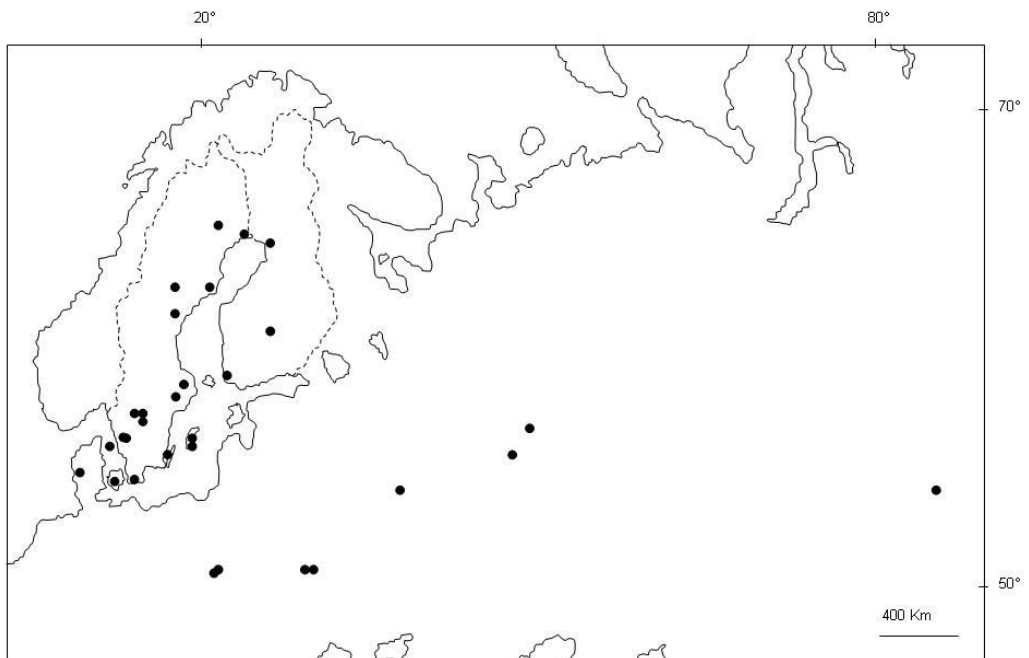


Figure 2. Approximate position of the 35 populations sampled for this thesis.

Molecular markers - an overview

Throughout this thesis the main molecular tools utilized are microsatellites. Additionally, in Chapter I the results are partly based on mtDNA sequences and in Chapter III quantitative genetic data is employed.

Microsatellites are sequences of DNA characterized by short (1-6 bp) nucleotide repeats of variable lengths (e.g. ACGACGA...CGACG). They are prevalent in the vertebrate genome and commonly display a high degree of polymorphism (Tautz 1989, Beckmann & Weber 1992). Combined with the ease of amplification at a relatively low cost, microsatellites have proven to be useful in various types of genetic studies. They have been applied to the identification of individuals (e.g. parentage analyzes) as well as in studies at the population level (population differentiation, effective population size, phylogeography etc; reviewed in Queller et al. 1993, Estoup & Angers 1998, Goldstein & Schlötterer 1998, Jehle & Arntzen 2002). However, though microsatellites are commonly used there are a few problems with them that should be taken into consideration. The first relates to the high mutation rate ($10^{-2} - 10^{-6}$ per locus per generation) that under the stepwise mutation model may lead to allele size homoplasy (Estoup et al. 1995). In other words, two alleles may be identical in size without being identical by descent. This in turn may lead to an underestimation of the population differentiation in a phylogenetic study. The other problem concerns non-amplifying alleles induced by mutations on the PCR

binding sequence. The presence of null-alleles may cause serious miss-assignments at the individual level and overestimate the rate of homozygosity in populations (Hoffman & Amos 2005). In my studies the populations are evolutionary young, thus minimizing the presence of homoplasy. The presence of null alleles has been checked with family data comprising offspring and both parents, thus segregations from the Mendelian inheritance could be detected. All populations were further tested for deviations from the Hardy-Weinberg equilibrium by comparing the expected (H_E) and observed (H_O) heterozygosities.

Mitochondrial DNA (mtDNA) has, over time, proven to be the most popular genetic marker in phylogeographic studies (Avice 2000). Like microsatellites, mtDNA is primarily neutral to selection (but see Hey 1997) and has a relatively high mutation rate. Additionally it is maternally inherited and lack recombination, which makes it suitable for recovering matrilinear genealogies. But like all markers, mtDNA has its limitations. When dealing with evolutionary young areas, the mutation rate of mtDNA might not be high enough to resolve the more recent phylogeographic lineages. Moreover, it reflects the history of effectively one locus, and thus, erroneous population genetic inferences are easily drawn (Pamilo & Nei 1988). Due to the maternal inheritance, incorrect inferences may also arise if dealing with species where the sexes have different migration rates.

Quantitative genetic variation

Quantitative genetics is a discipline that deals with the inheritance (i.e. heritability, h^2) of quantitative characters, such as body size, number of offspring, age at metamorphosis etc. Distinctive for these traits is their continuous rather than discrete distribution, and in particular their expression, which is influenced by several loci together with the environment. The major challenge in quantitative genetics is therefore to determine how much of the observed variation is inherited and how much is dependent on the environment (Frankham et al. 2002). The total phenotypic variation (V_P) in a trait can be divided into:

$$V_P = V_G + V_E + 2 \text{Cov}_{GE}$$

where V_G = genetic variance, V_E = environmental variance and 2Cov_{GE} = the covariance between genetic and environmental effects. The last term is expected to be zero if individuals are raised under the same environmental conditions, which usually is the case in crosses performed in laboratories (as opposed to wild populations). The genetic variance can be further partitioned into additive genetic variance (V_A), dominance variance (V_D) and interaction variance (V_I):

$$V_G = V_A + V_D + V_I$$

all of which possess significant information for conservational practices. By dividing V_A with V_P we obtain the heritability (h^2), which reflects the adaptive

evolutionary potential of the population for the character under consideration. V_D reflects the susceptibility to inbreeding depression and V_I determines whether outbreeding of the populations has beneficial or deleterious effects (Frankham et al. 2002). The combination of V_D and V_I is often referred to as non-additive effects.

Q_{ST} and its equivalent F_{ST} (for quantitative respectively neutral loci) are measures of population differentiation based on the comparison of genetic variability within and between populations. If F_{ST} is less than 0.05, then over 95 % of the variation is found within populations, and the interpopulation differentiation can be considered insignificant. By comparing Q_{ST} and F_{ST} , inferences can be made on the relative role that selection and drift have had in shaping the observed patterns (Merilä & Crnokrak 2001). Selection is expected to affect only Q_{ST} and thus, if selection is acting upon the trait under consideration we expect more (divergent selection) or less (stabilizing selection) differentiation in Q_{ST} in relation to F_{ST} . However, if genetic drift is the only factor operating there should be no difference between F_{ST} and Q_{ST} .

RESULTS AND DISCUSSION

The results of this study on *R. arvalis* conform to **I**) the hypothesis of a bi-directional recolonization of Northern Europe, **II**) the general pattern that amphibians exhibit a high degree of genetic differentiation between populations, and **III**) the conjecture that neutral marker genes may generally be quite uninformative about the levels of additive genetic variation in ecologically important traits. Moreover the results imply that **IV**) the males of this explosively breeding amphibian do not choose their lek according to kin and **V**) despite the commonly benign behaviour of the males, a genetic analysis of *R. arvalis* clutches of eggs revealed multiple paternity in the species. In what follows, I will discuss each of these issues in respect to published literature, and consider the broader significance of the results obtained. For the detailed results, see chapters I-V.

Genetic population structure, phylogeography and historical demography of Rana arvalis in Northern Europe

The postglacial history of Scandinavia

In Scandinavia, the prevalent determinant of the timing and phylogeographic patterning of species was the retreat of the final ice sheet situated in Northern Sweden (Lundqvist & Mejdahl 1995). It prevented movement between the western and eastern parts and hence, only when the ice melted, divergent genetic lineages were able to come into secondary contact. For

several terrestrial organisms studied, a common hybrid zone in Central and Northern Sweden has been identified (Fig. 1). Thus, a two-directional recolonization seems to be a frequent pattern among Scandinavian biota.

For *R. arvalis* the hypotheses of recolonization have earlier been based on the existence of several colour morphs in Scandinavia (Stugren 1966; Fog et al. 1997). Particularly the transition from one colour morph to another in Central Sweden has provoked the thought of a bi-directional colonization of *R. arvalis* to Fennoscandia. A glacial refugium in Eastern Russia has then been suggested (Stugren 1966). Babik et al. (2004) conducted a large-scale study of the mitochondrial phylogeography of *R. arvalis*. Based on these results, an origin in the Carpathian Basin was suggested for the majority of the western *R. arvalis* haplotypes. For the more eastern haplotypes, a refugium situated in South-eastern Russia - or even further east in Siberia or Kazakhstan - seems likely (Rafiński & Babik 2000; Babik et al. 2004). However, concerning the Scandinavian populations the resolution of these results was meagre.

In our study, including mtDNA and microsatellite data (**I**), mitochondrial haplotypes representing both the western and eastern lineages (*sensu* Babik et al. 2004) were found. Moreover, they were geographically structured supporting a bi-directional recolonization (Fig. 3). As these results were supported by the

Box 2. The main study questions and the corresponding results in the articles included in this thesis.

I	Does genetic data support a bi-directional recolonization of <i>R. arvalis</i> to Fennoscandia since the last glacial period?	All tests performed with microsatellites and mtDNA sequences clearly show a separation between east and west
	Are the phylogeographic patterns observed in colour morphs, nuclear genomes and mitochondrial DNA congruent?	The results based on genetic data were not supported by the phylogeographic distribution of the different colour morphs found. These may instead be influenced by environmental factors or natural selection.
II	What are the broad patterns of population substructuring in Scandinavia and are there variation in the levels of genetic diversity within local <i>R. arvalis</i> populations?	Despite their short evolutionary history, Scandinavian moor frog populations are clearly genetically structured. The amount of genetic variability increases up to the mid-latitudes, and declines dramatically thereafter. This could be a result of colonization history, or current demography with smaller populations in the north...
	Does the data support sex-biased dispersal in <i>R. arvalis</i> ?	No differences in dispersal patterns between the sexes could be observed.
	Are there any differences to the patterns of genetic structuring observed for the sympatric <i>R. temporaria</i> ?	The genetic structuring of these two species is in many ways similar, though some indications of contrasting interpopulation differentiation patterns could be distinguished in the comparisons.
III	What is the neutral genetic status of <i>R. arvalis</i> on Gotland as compared to other islands?	The genetic variability in <i>R. arvalis</i> populations on Gotland is severely reduced despite the large size of the island. These populations are also clearly differentiated from the mainland.
	Has inbreeding affected the levels of additive genetic variance in <i>R. arvalis</i> body size and tibia length in Gotland?	The reduced diversity of neutral genetic variation is not reflected in the additive genetic variation observed; on the contrary, the island population harboured relatively more additive genetic variation than the mainland population.
	Which have been the evolutionary forces shaping the observed population differentiation between Gotland and mainland?	The results suggest a selection for smaller size of <i>R. arvalis</i> on Gotland.
IV	Do <i>R. arvalis</i> males form their leks according to kin?	The males' relatedness within leks is not higher than expected by chance. Lek formation is suggested to have evolved by a female choice for breeding aggregations, combined with a female choice of habitat.
	Does a contrasting spatial configuration of leks affect the genetic relationship of the males?	The results for relatedness were similar in two types of habitat.
V	Is there multiple paternity in <i>R. arvalis</i> clutches of eggs?	Genetic data support the existence of multiple paternity in <i>R. arvalis</i> .

nuclear microsatellites, I feel quite confident to propose a bi-directional recolonization of *R. arvalis* to Scandinavia. Further, basing the inference on the geographic patterning of genetic divergence, and tests of individual assignment, I suggest the vast area around Vuollerim (66°26' N, 20°36' E) to be the hybrid zone where the diverse genetic lineages have met (Fig. 3).

Our results conform to the repeated pattern of a bidirectional colonization history of Scandinavia by various species (*Erinaceus europaeus*: Santucci et al. 1998; *Microtus oeconomus*: Brunhoff et al. 2006; *Fucus serratus*: Hoarau et al. 2007; *Ursus arctos*: Taberlet et al. 1995; *Cottus gobio*: Kontula & Väinölä 2001; *Betula pendula*: Palmé et al. 2003; *Vipera berus*: Carlsson et al. 2004; *Gasterosteus aculeatus*: Mäkinen et al. 2006).

In genetic terms the identification of several phylogeographic lineages has implications for conservational practices as well as ecological interpretations. Within the suture zone the inter-population genetic distances might be higher than expected by the geographic distances alone, which must be taken into consideration in the case of population management. The divergent genetic lineages may also display different ecological adaptations, and in extreme cases, interbreeding between the lineages might lead to a hybrid brake-down typical for interspecific hybridizations (Grant & Grant 2008). Regarding the geographic patterning of *R. arvalis* colour morphs (Fog et al. 1997), the genetic data rejects the hypothesis of different phylogeographic histories of the different phenotypes. The homologous distribution

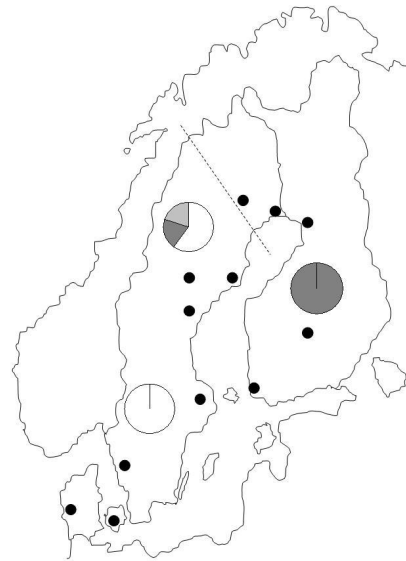


Figure 3. The geographic distribution of the three main mtDNA haplotypes in Fennoscandia. The thin dotted line represents the area where the western (white) and eastern (light + dark grey) haplotype lineages have met after the last glacial maximum.

of the *striata* form throughout northern Fennoscandia, and the finding of the *maculata* form both in southern Sweden and Finland, makes the phenotypic data incongruent to the genetic one. Therefore, either direct environmental influences (i.e. phenotypic plasticity) or natural selection acting on colouration may be responsible for the observed phenotypic patterning. This type of mismatch between genetic and phenotypic data is not unexpected, it is in fact, perhaps a rule rather than an exception (e.g. Leinonen et al. 2008, III).

Genetic divergence

In congruence with data from several other species (Hewitt 1999, Palo et al. 2004b),

our results indicate that northern *R. arvalis* populations have severely reduced genetic diversities (**I**, **II**, Fig. 4). However, in contrast to expectations, also the southern Swedish populations displayed low levels of genetic variation (**II**, Fig. 4). A reduction of genetic diversity towards the north is commonly explained by a series of founder events along the recolonization route (Hewitt 1996). But, the same pattern arises if populations are small and geographic (i.e. interpond) distances long, thus intensifying the effect of drift (Frankham et al. 2002). In large populations on the other hand, a reduced genetic diversity is commonly an indication of a population bottleneck in the past (Frankham et al. 2002, **III**). These examples point out the vast drawback of working solely with genetics; without ecological data it can be difficult to separate one explanation from the other to account for the observed pattern (**II**). Thus, in the case of the reduced variability in southern and northern populations, my inferences partly rely on the ecological observations made on *R. temporaria*.

Johansson et al. (2005, 2006) have showed that in southern Sweden the intensified agriculture has made *R. temporaria* to suffer from reduced inter-population connectivity. In the north on the other hand, Johansson et al. (2006) explained a reduced within-population genetic diversity, accompanied by increased among-population differentiation, in terms of smaller census population sizes and longer inter pond distances in northern as compared to more southern habitats. Though actual habitat/population data are missing from our studies, I believe the same ecological features along with the phylogenetic history of the populations are likely to explain the genetic patterning observed in *R. arvalis* (**II**). This conclusion is probably justified because the two species share many ecological features and often breed in the same ponds (Fog et al. 1997, Loman & Ladner 2006), even though some dissimilarities for instance in population dynamics can be seen (Loman & Andersson 2007).

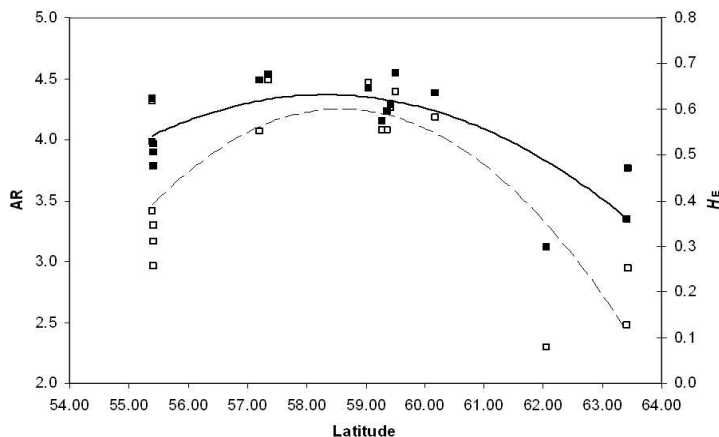


Figure 4. Latitudinal trends for *R. arvalis* in the measures of genetic diversity (■ H_E ; expected heterozygosity: $R^2 = 0.60$, $P = 0.002$; □ AR; allelic richness: $R^2 = 0.67$, $P < 0.001$).

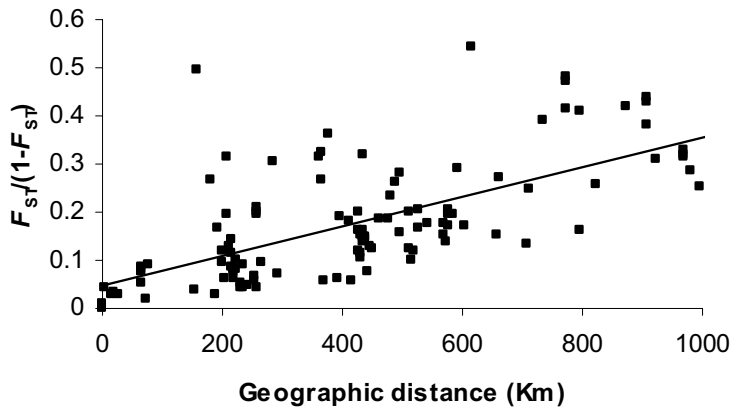


Figure 5. Isolation by distance in Scandinavian *R. arvalis* populations.

From a broader perspective, the results gained here are comparable to those obtained in other amphibian studies. Amphibians are well-known for being extremely site tenacious, as demonstrated by numerous capture-recapture studies (e.g. Haapanen 1970, Carpenter & Gillingham 1987; Gamble et al. 2007; Hamer et al 2008). From a genetic point of view, the strong homing behaviour preventing migration between populations will increase the rate of genetic drift. Consequently, in small populations the risk of extinction will be raised due to a loss of genetic variation. Additionally, the interpopulation diversity will increase, leading to significant population differentiations (as expressed by F_{ST}) between adjacent amphibian populations (e.g. Vos et al. 2001; Palo et al. 2004a).

Despite the comparatively young evolutionary history of Scandinavia, *R. arvalis* seems not to depart from this pattern (Fig. 5). Within Scandinavia, the average F_{ST} equalled 0.14 with regional

variations: at similar geographic distances the F_{ST} was higher in the northern (N) as compared to central (C) and southern (S) parts of Sweden (GLM; lsmeans $F_{ST(N)}=0.25$, $F_{ST(C)}=0.05$, $F_{ST(S)}=0.12$; Group: $F_{2, 40} = 42.55$, $P < 0.0001$; Group* Distance: $F_{2, 40} = 67.81$, $P < 0.0001$, **II**). For the local environmental authorities these results should act as guidelines in environmental planning. Due to the strong site fidelity of amphibians, their migration routes between over-wintering and lekking areas should not be prevented by barriers such as roads. Also, in order to facilitate migration, suitable lekking habitats should be retained at as short distances as possible from each other. Concerning *R. arvalis* (and *R. temporaria*; Johansson et al. 2005, 2006), the populations in Southern and Northern Sweden display a trend towards lost diversity and increased population differentiation and therefore, particular attention should be given to the amphibian habitats in these areas.

Neutral vs. additive genetic variation

Island populations often display extreme differentiations from their mainland counterparts, and this may facilitate and lead to speciation. Of the studies included in this thesis, one focused on the genetic variability and differentiation of *R. arvalis* populations on three large Baltic islands, with particular focus on Gotland (III). The genetic variability found in neutral marker genes was significantly reduced in all island populations as compared to mainland ones (unpaired t-tests; $t_s \geq 2.39$, $P \leq 0.033$), especially so in the geographically most isolated Gotland ($H_E = 0.16$).

A high degree of genetic differentiation between Gotland and the mainland populations was also observed ($F_{ST} = 0.31$, III). These findings are in line with theoretical expectations (e.g. Jaenike 1973, Frankham et al. 2002) and empirical data (Frankham 1998) which predict/indicate lowered genetic variability accompanied by increased genetic differentiation in small and isolated island populations.

The reduced genetic variability in neutral genes was not reflected in the variation in ecologically important traits as indicated by quantitative genetic breeding experiments. The additive genetic variation (V_A) in quantitative traits was not reduced, on the contrary, higher levels of V_A in body size and tibia length were found on the island of Gotland ($V_A = 16.697$ and 1.131 , respectively) as compared to the mainland population ($V_A = 4.517$ and 0.748 , respectively). Additionally, whilst a differentiation in neutral marker genes reflects primarily the effects of drift, a

possible differentiation in quantitative genetic traits could be attributable to drift, phenotypic plasticity, or selection. As the moor frogs on Gotland were phenotypically clearly differentiated from the mainland individuals (Fig. 6), we also compared the degree of differentiation in neutral marker genes (F_{ST}) with the differentiation in quantitative genetic traits (Q_{ST}) in order to elucidate the relative role that each of these evolutionary forces have had on the observed morphological divergence (i.e. if $F_{ST} < Q_{ST} \rightarrow$ directional selection; $F_{ST} > Q_{ST} \rightarrow$ stabilizing selection; $F_{ST} \sim Q_{ST} \rightarrow$ drift and/or selection; cf. Merilä and Crnokrak 2001).

Three different potential evolutionary scenarios were inferred (Fig. 7): while selection might explain a smaller size of *R. arvalis* on Gotland, the differentiation seen in absolute tibia length could be explained by genetic drift. The small difference in relative tibia length between Gotland and mainland would indicate stabilizing selection on this trait.

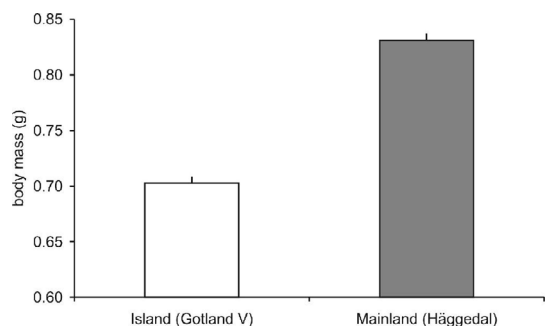


Figure 6. The mean body mass of *R. arvalis* metamorphs on Gotland and mainland as measured in standardised laboratory conditions.

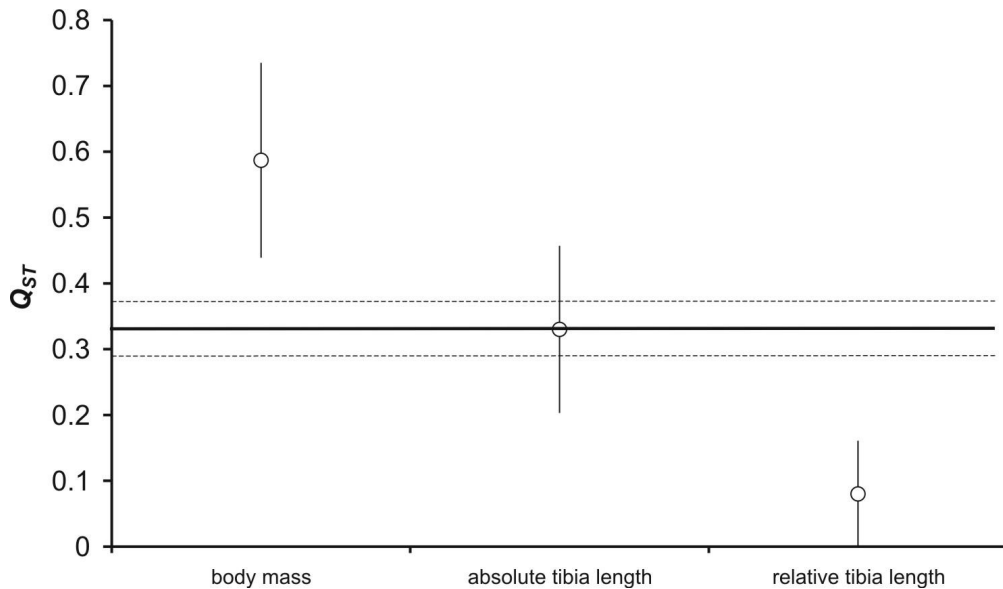


Figure 7. Q_{ST} (\pm S. E.) between Gotland and mainland populations for three traits measured (III). The horizontal line represents the F_{ST} (\pm S. E.) based on allelic variation in microsatellite loci between the same populations.

Overall the results indicate that *R. arvalis* on Gotland fulfil the conservational criteria of a separate management unit (MU, *sensu* Moritz 1994). Moreover, despite the wide utility of microsatellites in conservation biology, our results highlight the limited utility of neutral markers in predicting the amount of genetic variation in ecologically important quantitative traits, as well as in inferring the causes behind an observed phenotypic differentiation.

***Rana arvalis* leks – their genetic structuring and evolutionary significance**

The males of explosively breeding amphibians gather to a common mating ground in order to attract females. Within the mating ground the males typically form several smaller groups and the females will lay their eggs in close proximity to these male clusters. This kind of breeding

system is similar to the leks of birds (Hovi et al. 1994; Petrie et al. 1999; Krakauer 2005), some mammals (Clutton-Brock et al. 1993), insects (Downes 1969, Segura et al. 2007) and fish (Nordeide & Folstad 2000). However, though lekking is common among amphibians, little emphasis has been put on evaluating the selective pressures acting on the evolution of leks in this particular taxon.

Of the several theoretical models developed to explain the evolution of leks (e.g. Bradbury & Gibson 1983; Kokko 1997; Isvaran & St. Mary 2003), we chose the kin selection model as a starting point as its predictions are easily tested with genetic data (IV). The rationale behind this hypothesis is that by establishing themselves on a lek where the top male is a close relative, subordinate males may gain kin-selected benefits (Kokko & Lindström

1996) in addition to the occasional matings that the proximity of a preferred male might bring along. These benefits arise even if the subordinates do not get matings, but large leks as a whole attract more females than smaller ones. The larger leks may be more visible or audible (passive attraction, Parker 1983), or females may actively prefer to choose among many males (Kokko 1997).

For some species support has been given to the idea that kin selection may play a role in lek evolution (*Tetrao tetrix*: Höglund et al. 1999; *Pavo cristatus*: Petrie et al. 1999; *Manacus manacus*: Shorey et al. 2000; *Perca fluviatilis*: Gerlach et al. 2001; *Salmo trutta*: Carlsson & Carlsson 2002; *Meleagris gallopavo*: Krakauer 2005; *Plagiolepis pygmaea*: Trontti et al. 2005; *Tetrao urogallus*: Regnaut et al. 2006). However, the kin-selection hypothesis was not supported by our data on *R. arvalis* (IV): the degree of genetic structuring within populations was low (mean $F_{ST} = 0.005$, $P > 0.05$), as was the mean relatedness among males within leks ($0.38 < r < 0.48$). Also, there were equal numbers of probable full sibs within and between leks. These results were consistent in two habitats of differing spatial location of leks (leks in separate ponds vs. within the same water body). Hence, it seems that the clustering of *R. arvalis* males is random in respect to their relatedness.

On the basis of results gained and general knowledge of anuran breeding biology, I suggest that lek formation in explosively breeding amphibians might have evolved by a female choice for breeding aggregations, combined with a female choice of habitat (IV, V). A strong

correlation has been shown between lek attendance and mating success (Fiske et al. 1998; Friedl & Klump 2005). This leads to a selection pressure for males to spend as much time as possible on the lek, where additionally direct male-male competition may occur (Wells 1977). Moreover, assuming that lek attendance is a condition-dependent trait (e.g. Kokko et al. 1999, Sullivan & Kwiatkowski 2007), females that mate on leks should have a high probability of mating with a good quality male (even if mate choice within the lek is random; Friedl & Klump 2005). The male assemblages are additionally likely to reduce the time required for searching for a mate. This is highly important for the explosive breeders due to the short time given for larval development. Eggs laid too late may suffer from reduced survival probability due to a precipitated development at the larval stage (e.g. Loman 2002). For the same reasons, moor frogs may choose to mate on warmer sunny patches, where the eggs will develop faster. If a preferred habitat is saturated, it becomes beneficial to establish a new lek. Also, at large patches the per capita mating prospects will decrease if the female visitation rate fails to increase in proportion with lek size (Hutchinson 2005). Then it may be beneficial for the males to form smaller groups within reasonable travel distances for females.

From a female point of view, lekking may theoretically also increase fertilization success, sperm quality, and/or genetic diversity of the offspring through multiple paternity (Jennions et al. 2000; Vieites et al. 2004) Reichard et al. 2007; Sagvik et al 2008; but see Byrne & Roberts 1999) (V).

For many explosively breeding amphibians, the operational sex ratio (OSR) is strongly male-biased at the lek (Wells 1977, Lodé et al. 2005). This leads to fierce competition among the males and in order to reproduce, they may form “mating balls” consisting of several males amplexing one female. Once the female releases her eggs, a sperm competition may take place and lead to multiple paternities within the affected clutch of eggs (V).

Lately, it has become clear that multiple paternity is more prevalent in amphibians than earlier has been anticipated. However, the proximate reason of multiple paternity has remained a matter of debate and its evolutionary significance has been poorly examined in this taxon. Multiple paternity has been suggested to be the result of active multiple mating (Sztatecsny et al. 2006), clutch piracy (Vieites et al. 2004) or sperm leakage at the communal lek (Laurila & Seppä 1998).

Compared to other explosively breeding anurans *R. arvalis* males are exceptional in that multiple amplexi are seldom observed. According to own observations (confirmed by a few other herpetologists) the pairs in amplexus are usually left alone and mating can take place undisturbed. Therefore we would expect *R. arvalis* to have lower levels of multiple paternity than observed for the sympatric *R. temporaria* (~50% of clutches, Laurila & Seppä 1998) and *Bufo bufo* (~30 %, Sztatecsny et al. 2006). This was however not the case (V). Our results suggest that though present at slightly lower frequencies than reported for the other two species, multiple paternity can

be found at relatively high frequencies both among (14-29%) and within clutches (23%) also in *R. arvalis*.

In line with earlier work, our study design mainly answers the question on whether or not there is polyandry in the species. Thus, both the proximate and ultimate reason behind multiple paternity remains yet unknown. There are, however, several theoretical expectations that could be tested in future work in order to understand the evolutionary significance of multiple paternity in amphibians. For instance, it is known that OSR and population density may affect the mating behaviour of anuran males (Arak 1983, Lodé et al. 2005). If the rate of multiple mating increases in dense populations and results in higher rates of polyandry, then multiple paternity could be considered mainly as an attempt of the males to maximize their fitness. If on the other hand the primarily purpose of multiple paternity is to increase the genetic diversity of the offspring, we would expect higher rates of polyandry in smaller (increased risk of inbreeding) vs. larger populations. Finally, if the proximate reason of multiple paternity is mainly sperm leakage in the waterbody instead of active multiple matings, we may expect higher rates of polyandry in the centre of the communal lek vs the peripheral areas. This topic is also interesting from the point of view that it enhances the role of both the position of individual males and clutches of eggs within leks.

CONCLUSIONS AND FUTURE DIRECTIONS

The main aim of this thesis was to investigate the phylogeographic history and broad patterns of population subdivision of *R. arvalis* in Northern Europe. The results add information to our understanding of amphibian population structure in general, and particularly that of the Scandinavian fauna. For the local environmental authorities the results might be helpful by providing information needed for the management of *R. arvalis* and its habitats.

In chapter I a bi-directional recolonization of Fennoscandia by *R. arvalis* was confirmed. The results based on genetic data were not supported by the phylogeographic distribution of the different colour morphs found in the species. Thus, the hypothesis based on phenotypic distribution according to population history, as suggested earlier, may be rejected. Future studies should focus instead on the selective and environmental influences on colouration. In chapter II and III the regional differences in local genetic diversities and interpopulation differentiations were clearly demonstrated. This kind of large-scale study has not been conducted earlier with *R. arvalis* and the results should be useful in future conservational practices and environmental plans concerning this protected species. Concerning the island of Gotland, the results indicate that *R. arvalis* fulfil the conservational criteria of a separate management unit (MU, *sensu* Moritz 1994) and should be protected accordingly.

In chapter IV we focused on the selective pressures acting on the evolution of leks in

amphibians; a subject seldom discussed earlier. Our tests on lekking according to kin gave negative results, but this should anyhow act as a good starting point for future studies. Especially the differences between explosively and prolonged breeding amphibians should be an interesting topic. Also concerning multiple paternity in amphibians (V) the two breeding systems may display contrasting patterning as more studies hopefully will emerge. The proximate (and ultimate) cause behind multiple paternity in *R. arvalis* and other amphibian species also remains a subject for future studies.

Finally, having detailed background knowledge on population connectivity and heterogeneity it is easier to elucidate or diminish the effects of anthropogenically induced changes in the environment. However, despite the wide utility of microsatellites in conservation biology, our results highlight the limited utility of neutral markers in predicting the amount of genetic variation in ecologically important quantitative traits (III).



W.K.

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