Living in a naturally fragmented world: from extra-pair paternity in local populations to spatial population structure of the reed bunting (Emberiza schoeniclus) across Europe

Mayer, Christian

Abstract: Species often occur in subdivided populations as a consequence of spatial heterogeneity of the habitat. Destruction and fragmentation of habitats due to human land use generally decrease the size and the connectivity of subdivided populations. Due to deterministic and stochastic effects on demographic and genetic parameters, long-term persistence of subdivided populations may be compromised. Theoretical studies have shown that viability of subdivided populations critically depends on the connectivity among local subpopulations, and population models have been developed which predict extinction risks of subpopulations in relation to different levels of connectivity. The lack of empirical data on how subpopulations are spatially organized makes it difficult to judge the general validity of theoretical population models. In fact, predictions derived from population models have rarely been tested with empirical data, although conservation biology focuses on preventing declines and extinctions of populations. However, traditional population definitions are often simplistic, being based for example on the size of a habitat fragment or the geographic clustering of individuals into different breeding units. These population definitions neglect the genetic boundaries of populations if gene flow occurs. The theoretically predicted positive relationship between population size and genetic diversity, which has been supported by many empirical studies, may therefore not be found if gene flow connects populations. Thus, population size may not allow conclusions about the genetic diversity of local populations, which has implications for conservation management of small populations. However, when studying animal behaviour it is useful to focus on populations defined as groups of individuals that have the opportunity to interact in a particular area at a particular time. According to traditional definitions, species living in habitat fragments scattered throughout the landscape occur in geographically distinct local populations and provide the opportunity to test the generality of behaviours by comparing multiple local populations. An example for a species inhabiting naturally fragmented habitat is the reed bunting (Emberiza schoeniclus), a small Palearctic short-distance migratory songbird restricted to wetlands. Wetlands have been destroyed worldwide due to anthropogenic land use (Keddy 1999). Between the 1970s and early 1990s, considerable declines of the reed bunting have been reported for several European countries, for example Germany, Belgium, Finland and England (Heath et al. 2000). Especially in the intensively used agricultural landscapes of south and west Central Europe, habitat destruction has led to extinction of local populations (Glutz von Blotzheim Bauer 1997) and to reductions in local population sizes (Blümel 1995). The reed bunting is therefore an ideal study species to evaluate the significance of small local populations in population networks and to assess the importance of local population size for the maintenance of genetic diversity in this species. The occurrence of the species in geographically distinct breeding units also provides the opportunity to evaluate the significance of ecological parameters explaining mating-system differences among local populations. A precondition for the analyses of the spatial distribution of genetic variation in subdivided populations, as well as for the analysis of paternity in avian mating-systems, is the availability of polymorphic molecular markers. In chapter 1, I characterized fifteen microsatellite loci specifically designed for the reed bunting. Eleven loci were autosomal and four linked to the Z-chromosome. All loci were characterized and tested in 45 unrelated reed buntings from a Swiss population. Autosomal and z-linked loci were highly polymorphic allowing the inference of spatial genetic structure and the analysis of paternity in reed buntings. In chapter 2, I described the spatial organization of a local system of reed bunting

subpopulations. Existing theory proposes three main population models, which predict different levels of connectivity among and extinction risks of subpopulations: patchy population, metapopulation and isolated populations. However, spatially discrete subpopulations are commonly considered to be organized as metapopulations, although explicit tests of metapopulation assumptions are rare. I tested predictions of the three models on the basis of demographic and genetic data, a combined approach so far surprisingly little used in mobile organisms. From 2002-2005, I studied nine subpopulations of the wetland- restricted reed bunting in the southeastern part of the Canton Zurich (Switzerland), from where local declines of the species had been reported. Here, wetlands can be as small as 2.7 ha, often host very few breeding pairs and are separated through intensively used agricultural landscapes. Demographic data referred to dispersal of colour-banded individuals among subpopulations, immigration rates and extinction-/recolonization dynamics. Genetic data were based on the distribution of genetic variability and gene flow among subpopulations derived from the analysis of nine microsatellite loci. Both demographic and genetic data revealed that the patchy population model best described the spatial organization of reed bunting subpopulations. High levels of dispersal among subpopulations, high immigration into the patchy population, and genetic admixture suggested little risk of extinction of both subpopulations and the entire patchy population. This study exemplifies that spatially discrete subpopulations may be organized in ways other than a metapopulation, which has implications for the conservation of subpopulations and species. In chapter 3, I investigated the importance of local population sizes for the maintenance of genetic diversity in the reed bunting. Reduction of genetic diversity due to genetic drift depends on population effective size, which is often correlated with local population size, i.e. population census or breeding size. However, if gene flow is high, the genetic boundaries of a local population may greatly exceed the geographic area originally used for population delineation. As a consequence, the predicted positive relationship between local population size and genetic diversity may not be found. In reed buntings, the distribution and size of local populations is restricted according to the distribution and size of wetlands. I show that genetic diversity of local reed bunting populations depended on regional abundance of the species rather than on local population sizes, which is in accordance with the high level of gene flow we found among reed bunting populations across Europe. Genetic diversity increased with latitude and was highest in Scandinavia (samples from Norway, located along the presumed centre of the species' distribution range), and lowest in south-central Europe (samples from Switzerland, located along the southern edge of its distribution range). At the species' southern range margin, increased temporal variability in population sizes may have reduced population long-term effective sizes and thus genetic diversity. A further reduction of genetic diversity may be counterbalanced if conservation efforts focus on the protection of remaining wetlands, enhancing habitat quality and thus ultimately population sizes. In chapter 4, I studied the relation between population density and variation of extra-pair paternity (EPP) in reed buntings. Density has been suggested to affect variation of EPP within avian species, because it increases encounter rates and mating opportunities when individuals search for potential extra-pair mates. So far, the significance of density affecting EPP variation in intraspecific comparisons is controversial. However, the absence of a relationship between density and EPP in within- and among-population comparisons as revealed by many empirical studies may mostly be attributed to potentially confounding factors and poor study design. Density measures may not always reflect extra-pair mating opportunities, mate guarding efforts may vary with density, different migration distances and climatic conditions could cause population differences in EPP, and low variation in density and small sample sizes weaken the test power. Taking all those factors into account, I tested if EPP rates within and among local reed bunting populations were related to density. My analyses were based on data from 18 local populations studied over four years. Within populations, the proportion of extra-pair young (EPY) in broods was positively related to local breeding density. Similarly, among local populations, proportion of EPY was positively associated with population density. I also show that EPP was absent where populations consisted of a single breeding pair, i.e. when no extra-pair mating opportunities were available. My study confirms that density is an important biological factor, which significantly influences the amount of EPP within and among populations, but also supports the view that additional mechanisms contribute to EPP variation. Zusammenfassung Als Konsequenz aus der räumlichen Heterogenität ihres Habitats kommen Arten oft in fragmentierten Populationen vor. Zerstörung und Fragmentation des Habitats durch anthro- pogene Nutzung verringern nicht nur die Grösse, sondern auch die Vernetzung fragmentierter Populationen. Das Langzeitüberleben fragmentierter Populationen ist aufgrund determin- istischer und stochastischer Effekte, die Demographie und Genetik fragmentierter Popula- tionen stark beeinflussen können, möglicherweise vermindert. Theoretische Studien haben gezeigt, dass das Langzeitüberleben fragmentierter Populationen stark von der Vernetzung lokaler Subpopulationen abhängt, und es wurden Populationsmodelle entwickelt, die die Vernetzung und das Aussterberisiko solcher Subpopulationen vorhersagen. Die generelle Aussagekraft theoretischer Populationsmodelle lässt sich aber nur schwer feststellen, da wenig empirische Daten zur Verfügung stehen. Die Voraussagen theoretischer Populations- modelle wurden bisher auch nur selten getestet, obwohl ein ganzer Forschungsbereich, die Naturschutzbiologie, einen ihrer Schwerpunkte darin sieht Bestandsabnah-

men und das Aussterben von Populationen zu verhindern. Traditionelle Definitionen einer Population sind in der Regel vereinfachend, da sie beispielsweise Individuen aufgrund geographischer Gegebenheiten in verschiedene Gruppen aufteilen. Diese traditionellen Populationsdefinitionen beachten dabei nicht, dass Populationen über Genfluss miteinander in Verbindung stehen können. Die Grösse einer Population wird häufig als Mass dafür genommen, ob eine Population ausreichend genetisch divers ist, um langfristig überleben zu können. Für durch Genfluss vernetzte Populationen hätte die theoretische Vorhersage, dass die genetische Diversität einer Population von ihrer Grösse abhängt, keine Gültigkeit mehr, woraus sich Folgerungen für den Schutz kleiner Populationen ableiten lassen. In Abhängigkeit vom biologischen Kontext einer Studie oder Fragestellung können traditionelle Populationsdefinitionen aber durchaus sinnvoll sein. Wenn zum Beispiel das Verhalten von Tieren untersucht wird, ist es sinnvoll eine Population so zu definieren, dass sie die Interaktionen von Individuen an einem bestimmten Ort und zu einer bestimmten Zeit umfasst. Ausgehend von traditionellen Populationsdefinitionen kommen Arten, die in fragmentierten Landschaften leben, in geographisch distinkten lokalen Populationen vor. Dies bietet die Möglichkeit Verhalten in verschiedenen Populationen zu untersuchen und zwischen verschiedenen Populationen zu vergleichen. Ein Beispiel für eine in natürlich fragmentiertem Habitat vorkommende Art ist die Rohrammer (Emberiza schoeniclus). Diese paläarktisch verbreitete Singvogelart ist ein Kurzstreckenzieher dessen Vorkommen sich auf Feuchtgebiete beschränkt. Feuchtgebiete wurden und werden durch anthropogene Nutzung weltweit zerstört. Aufgrund dessen nahm zwischen den Siebziger und Neunziger Jahren des letzten Jahrhunderts der Rohrammer- bestand in verschiedenen europäischen Ländern wie Deutschland, Belgien, Finnland oder England ab. Besonders in den südlichen und westlichen, landwirtschaftlich intensiv genutzten Teilen Mitteleuropas hat die Zerstörung der Feuchtgebiete zu Bestandsabnahmen und zum Aussterben lokaler Rohrammerpopulationen geführt. Aufgrund ihrer Habitatspezifität ist die Rohrammer eine ideale Art, um die Bedeutung kleiner Populationen in Populationsnetzwerken sowie die Bedeutung von Populationsgrössen für den Erhalt genetischer Diversität zu untersuchen. Dass die Rohrammer in geographisch distinkten Gruppen brütet, ermöglicht es ausserdem den Einfluss ökologischer Parameter auf Aspekte des Paarungssystems der Rohrammer zwischen verschiedenen geographisch distinkten Populationen zu vergleichen. Eine Voraussetzung für derartige Analysen ist die Verwendung polymorpher molekularer Marker. In Kapitel 1 charakterisiere ich fünfzehn Mikrosatellitenloci, die spezifisch für die Rohrammer entwickelt wurden. Elf dieser Loci waren autosomal, die restlichen vier geschlechtsspezifisch (d.h. sie lagen auf dem Z-Chromosom). Alle Loci wurden in 45 nicht miteinander verwandten Rohrammern einer schweizer Population charakterisiert und getestet. Sowohl die autosomalen als auch die z-gelinkten Loci waren hochpolymorph, was die Untersuchung der räumlichen genetischen Struktur von Rohrammer- populationen und von Vaterschaftsanalysen möglich machte. In Kapitel 2 beschreibe ich die räumliche Organisation eines lokalen Systems von Rohrammersubpopulationen. Aus der Theorie sind drei Populationsmodelle bekannt, die Voraussagen über Vernetzung und Aussterbeereignisse lokaler Subpopulationen machen. Herkömmlicherweise nimmt man für geographisch diskrete Subpopulationen eine Metapopulationsstruktur an. Tests, die überprüfen, ob die räumliche Organisation der Subpopulationen die Annahmen einer Metapopulation auch erfüllt, sind allerdings selten. Ich testete die Voraussagen dreier Populationsmodelle auf der Grundlage demographischer und genetischer Daten. Dieser kombinierte Ansatz wurde bis jetzt erstaunlich selten gewählt, zumindest für mobile Organismen. Zwischen 2002 und 2005 untersuchte ich neun Subpopulationen der Rohrammer im südöstlichen Teil des Kantons Zürich (Schweiz), von wo vereinzelt Bestandsrückgänge der Art beschrieben wurden. In diesem Teil des schweizerischen Mittellandes sind Feuchtgebiete teilweise nur 2.7 ha gross und durch landwirtschaftlich intensiv genutzte Flächen voneinander getrennt. Farbberingte Individuen lieferten demographischen Daten über die Dispersion zwischen Subpopulationen, Immigrationsraten und Aussterbe- und Wiederbesiedlungsereignissen. Die genetischen Daten basierten auf der molekularen Information von neun Mikrosatellitenloci und beschreiben die Verteilung genetischer Variation sowie den Genflusses zwischen den Subpopulationen. Sowohl die demographischen als auch die genetischen Daten zeigen, dass die Organisation der Subpopulation am besten durch das Modell der 'Patchy population' beschrieben wird. Das hohe Mass an Dispersal zwischen den Subpopulationen, die hohe Immigrationsrate in die gesamte 'Patchy population' und die fehlende genetische Differenzierung zeigen, dass sowohl für die einzelnen Subpopulationen als auch für die gesamt 'Patchy population' nur eine geringe Aussterbewahrscheinlichkeit besteht. Diese Studie ist ein Beispiel dafür, dass geographisch diskrete Subpopulationen räumlich durchaus anders als Metapopulationen organisiert sein können, woraus sich bestimmte Folgerungen für den Schutz von Subpopulationen und Arten ableiten lassen. In Kapitel 3 untersuchte ich die Bedeutung lokaler Populationsgrössen für den Erhalt genetischer Diversität am Beispiel der Rohrammer. Die Verringerung genetischer Diversität durch genetische Drift hängt generell von der effektiven Populationsgrösse ab, die oft mit der lokalen Populationsgrösse korreliert ist. Lokale Populationsgrössen werden meist als die Anzahl der lokalen Individuen oder als die Anzahl brütender Individuen angegeben. Wenn Genfluss zwischen lokalen Populationen hoch ist, kann die genetische Abgrenzung einer lokalen Population allerdings den ursprünglich für die Populationsdefinition benutzten lokalen geographischen Rahmen bei

weitem überschreiten. Der theoretische vorhergesagte Zusammenhang zwischen lokaler Populationsgrösse und dem Ausmass genetischer Diversität kann dadurch möglicherweise nicht mehr gefunden werden. Bei der Rohrammer sind lokale Populationsgrössen von der Verteilung und der Grösse von Feuchtgebieten abhängig. In dieser Studie zeige ich, dass aufgrund hohen Genflusses die genetische Diversität lokaler Rohrammerpopulationen von der regionalen Dichte der Rohrammer und nicht von den lokalen Populationsgrössen abhängt. Mit zunehmendem Breitengrad nahm die genetische Diversität zu und war am höchsten in Skandinavien (Norwegen) und am niedrigsten am südlichen Rand des Rohrammerverbreitungsgebiets (Schweiz). Temporäre Veränderungen der lokalen Populationsgrössen über längere Zeiträume hinweg könnten dazu geführt haben, dass die durchschnittlichen effektiven Populationsgrössen und damit auch die genetische Diversität am südlichen Rand des Verbreitungsgebiets abnahmen. Der weiteren Abnahme genetischer Diversität könnte allerdings durch geeignete Schutzmassnahmen entgegengewirkt werden, wenn diese Massnahmen eine Qualitätsverbesserung des Rohrammerhabitats und damit letztlich auch eine Vergrösserung der lokalen Populationen zur Folge haben. In Kapitel 4 untersuchte ich den Einfluss der Brutdichte auf die Häufigkeit von Fremdvaterschaften bei der Rohrammer. Theoretisch gesehen führt zunehmende Dichte einerseits dazu, dass sich Männchen und Weibchen ausserhalb des sozialen Paarbundes häufiger begegnen. Andererseits ergeben sich mit zunehmender Dichte generell mehr Möglichkeiten einen potentiellen Partner für das Fremdgehen zu finden. Allerdings hat sich bisher die Bedeutung der Dichte als Erklärung für die hohe Variation im Ausmass von Fremdvaterschaften zwischen verschiedenen Populationen derselben Art nicht als sehr überzeugend herausgestellt. Dass viele Studien keinen Zusammenhang zwischen Dichte und der Häufigkeit von Fremdvaterschaften gefunden haben ist möglicherweise eine Folge ihres methodischen Aufbaus, beziehungsweise der Nichtberücksichtigung von Faktoren, die das Untersuchungsergebnis beeinträchtigt haben könnten. Die in diesen Studien verwendeten Masse für Dichte spiegelten nicht immer die Möglichkeit des Fremdgehens wider. Unterschiedliche Migrationsdistanzen und klimatische Verhältnisse zwischen Populationen könnten ebenfalls Ergebnisse verfälscht haben. Hinzu kommt, dass sowohl eine zu geringe Bandbreite an Dichten, unter denen die Häufigkeit von Fremdvaterschaften gemessen wurde, als auch eine zu geringe Stichprobengrösse die Aussagekraft der verwendeten Tests verringert haben könnten. Unter Berücksichtigung all dieser Faktoren testete ich, ob es einen Zusammenhang zwischen der Häufigkeit von Fremdvaterschaften innerhalb und zwischen verschiedenen lokalen Rohrammerpopulationen und der jeweiligen Dichte gab. Meine Analysen basierten auf Daten von 18 lokalen Populationen, die während vier Jahren untersucht wurden. Innerhalb der Populationen war die Häufigkeit von Fremdvaterschaften positiv mit der lokalen Brutdichte korreliert. Beim Vergleich zwischen verschiedenen Populationen waren Häufigkeiten von Fremdvaterschaften positiv mit der Populationsdichte korreliert. In lokalen Populationen, die nur aus einem einzigen Brutpaar bestanden und in denen es somit keine Möglichkeit des Fremdgehens gab, kam es auch nie zu Fremdvaterschaften. Damit bestätige ich in meiner Studie die Hypothese, dass Dichte tatsächlich ein biologisch relevanter Faktor ist, der einen signifikanten Einfluss auf die Häufigkeit von Fremdvaterschaften innerhalb und zwischen verschiedenen Populationen hat. Darüber hinaus bestätigen meine Ergebnisse die Ansicht, dass neben der Dichte weitere Mechanismen die Häufigkeit von Fremdvaterschaften beeinflussen.

Living in a Naturally Fragmented World: From Extra-pair Paternity in Local Populations to Spatial Population Structure of the Reed Bunting (*Emberiza schoeniclus*) across Europe

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Prof. Dr. Heinz-Ulrich Reyer (Vorsitz)

Dr. Gilberto Pasinelli (Leitung)

Prof. Dr. Lukas Keller

Prof. Dr. Jan Komdeur

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GENERAL INTRODUCTION

Species often occur in subdivided populations as a consequence of spatial heterogeneity of the habitat. Heterogeneity of the habitat may have two reasons (Fischer & Lindenmayer 2007). First, the habitat has a naturally fragmented distribution or second, formerly continuous habitats have been destroyed and subdivided into fragments due to anthropogenic landscape modifications. Because the ongoing destruction and fragmentation of habitats due to human land use generally reduces the size and the connectivity of the remaining local populations, species having evolved both in continuous habitats and in naturally fragmented habitats may be affected (Frankham *et al.* 2002).

From a conservation perspective, the ongoing destruction and fragmentation of habitats resulting in increased population subdivision and reduction of population sizes is alarming. In general, the long-term persistence of subdivided and small populations is reduced due to deterministic and stochastic effects on demographic parameters. Furthermore, subdivision and small population sizes intensify the effects of genetic stochasticity leading to loss of genetic variation through random genetic drift (Frankham *et al.* 2002) and loss of fitness due to inbreeding (Frankham 1995; Keller *et al.* 2002). The impact of both demographic and genetic factors on population persistence are predicted to depend on time since fragmentation, the size and the spatial distribution of populations. In this respect, gene flow and connectivity among populations appear to be critical factors influencing the genetic structure and demography of subdivided populations, especially when they are small in size (Gilpin & Hanski 1991; Harrison 1991).

Theoretical studies have shown that viability of populations critically depends on the connectivity among local populations, usually referred to as spatial organization of populations (Gilpin & Hanski 1991). For example, spatially discrete populations have an increased probability to survive when they are organized as metapopulations (Gilpin & Hanski 1991). However, the term metapopulation is currently often used to refer to a set of spatially discrete local populations within a fragmented landscape in general (Hanski & Gaggiotti 2004). This view is challenging, because alternative population models exist which describe the spatial organization of populations more differentiated (for example Harrison 1991). Furthermore, to refer to all sets of local populations generally as 'metapopulations' does not provide more information about their spatial organization and long-term persistence than simply calling them 'fragmented' or 'subdivided' populations, as has been done before. However, the lack of empirical data on spatial structure, especially of small populations,

makes it difficult to judge the general validity of theoretical population models. That predictions derived from theoretical populations models have rarely being tested empirically is surprising, because species conservation specifically focuses on halting declines in and preventing extinctions of local populations. From a conservation perspective, thus, more research is needed that focuses on population dynamics and genetics to design networks of suitable habitat allowing the long-term persistence of populations in fragmented landscapes. This need especially exists for species occurring in naturally fragmented habitats because for these species the effects of habitat loss and fragmentation are even less studied than for species in previously continuous habitats, despite the ongoing destruction of naturally fragmented habitats, e.g. wetlands.

Conservation of genetic diversity is a fundamental concern in conservation biology. Genetic diversity is the raw material for evolutionary change, and evolvability of natural populations may be restricted when genetic diversity is reduced (Spielman *et al.* 2004). Theory predicts genetic diversity to be related to population effective size (Hedrick 2005). Population census size has often been used as a proxy for population effective size. In fact, many studies revealed a positive relationship between population census size and levels of genetic diversity in natural populations (reviewed in Frankham 1996). However, if gene flow among populations occurs, population census sizes may not always correlate with population effective sizes (van Rossum *et al.* 1997). This is due to the fact that traditional population definitions often rely on qualitative descriptors that do not take the genetic boundaries of populations into account (for review see Waples & Gaggiotti 2006). For example, when individuals breed within geographically distinct habitat patches, which is particularly the case for species occurring in naturally fragmented habitats, it is tempting to define populations according to the distribution of geographically distinct breeding units. Population census size may therefore not allow conclusions about the genetic diversity of local populations, which has implications for conservation management of populations with small census sizes. Assessing the importance of population sizes for the maintenance of local genetic diversity requires data on genetic diversity and gene flow among multiple populations over extensive geographic scales. This assessment is necessary to identify the genetic extent of populations and to determine the geographic scale needed to apply effective conservation measures.

However, there is no single correct general answer to the question which population definition is useful, because population definitions may depend on the underlying objectives of a study or question. In contexts different from conservation biology, traditional population definitions, which do not consider genetic boundaries, also have their applications and

advantages. For example, when studying behavioural interactions in animals, it is useful to define populations according to groups of individuals that have the opportunity to interact in a particular area at a particular time (for example Krebs 1994; Lapedes 1978). Testing the generality of behaviours requires comparisons among different populations of the same species (Griffith *et al.* 2003). According to traditional population definitions, species living in highly fragmented landscapes occur in geographically distinct local populations. These species, therefore, provide a good opportunity to study behaviour among multiple populations with homogenous geomorphological and climatic conditions.

Mating strategies have often been the focus of behavioural studies in birds. The most important discovery with respect to avian mating systems was that extra-pair paternity occurs regularly in over 80 % of all passerine bird species (Griffith *et al.* 2002) that had formerly been classified as monogamous (Lack 1968). Extra-pair paternity (EPP) is defined as fertilization resulting from copulations outside the social bonds recognized by the traditional mating system classification (Westneat *et al.* 1990). Comparisons in EPP rates among various avian species as well as among different populations of the same species have raised the question of why there is so much variation in the rate of EPP. One of the traditional explanations for inter- and intraspecific variation in EPP rates states that EPP rate is positively related to breeding density ('density hypothesis', see Westneat *et al.* 1990). While there is little evidence of a general interspecific relationship between breeding density and EPP rates, the intraspecific situation is less clear (Griffith *et al.* 2002). It has been suggested that breeding density may explain differences between individuals within populations and possibly variation between different populations of the same species (Griffith *et al.* 2002), but so far results are puzzling (Westneat & Stewart 2003). Data on intraspecific comparisons among populations often originated from studies that were not explicitly designed to test the density hypothesis. Since usually only two populations were compared, intraspecific analyses on variation in EPP suffered from insufficient statistical power. To evaluate the importance of breeding density explaining intraspecific variation in EPP, data are needed that include multiple populations of the same species.

An example for a species inhabiting naturally fragmented habitat is the reed bunting (*Emberiza schoeniclus*). The reed bunting is a small Palearctic short-distance migratory songbird restricted to wetlands. Wetlands have been destroyed worldwide due to anthropogenic land use (Keddy 1999). Between the 1970s and early 1990s, considerable declines of the reed bunting have been reported for several European countries, for example Germany, Belgium, Finland and England (Heath et al. 2000). Especially in the intensively

used agricultural landscapes of south and west Central Europe, habitat destruction has led to extinction of local populations (Glutz von Blotzheim & Bauer 1997) and to reductions in local population sizes (Blümel 1995), possibly making small local populations vulnerable to erosion of genetic diversity. The reed bunting is an ideal study species to evaluate the significance of small local populations in population networks and to assess the general validity of spatial population models. The geographic distinctiveness of populations allows to assess the importance of local population size for the maintenance of genetic diversity in this species and to critically analyse the use of traditional population definitions in conservation biology. The reed bunting is also a promising species to study intraspecific variation in EPP, since its occurrence within highly fragmented landscapes allows comparisons of multiple local populations with varying breeding densities on logistically reasonable spatial scales.

Research topics addressed in this thesis

Polymorphic molecular markers and powerful statistical methods allow the investigation of the spatial distribution of genetic variation in fragmented populations and provide a measure of population connectivity. Molecular markers also allow determining paternity when mating systems are studied. Since molecular markers were required to answer my research questions, in chapter 1, I first characterized 15 highly polymorphic microsatellite markers that were specifically isolated for reed buntings. I designed multiplex PCRs that were then used to assess the spatial distribution of genetic variation in reed bunting populations (chapter 2 and 3) as well as to determine paternity in mating system analyses (chapter 4).

In chapter 2, I characterized the spatial structure of local reed bunting populations by testing predictions of theoretical population models. These population models predict different levels of connectivity among and extinction risks of local subpopulations. I tested these predictions on the basis of demographic and genetic data, that is direct and indirect estimates of dispersal and gene flow, because they yield information on current and past gene flow.

The geographic boundaries of wetlands are often used to define local reed bunting populations, which are then considered as conservation units. However, it is unknown whether these local populations are genetically distinct given the high dispersal capacity of reed buntings. By extending the local to the regional geographic scale, I investigated the importance of local population sizes and the impact of gene flow on the distribution and maintenance of genetic diversity in local reed bunting populations across Europe (chapter 3).

In chapter 4, I made use of traditional population definitions and compared the extra-pair mating behaviour of reed buntings breeding in different local populations. I tested two predictions of the density hypothesis. First, I predicted that levels of EPP within populations were positively related to local breeding density, assessed through measures at the territory level. Second, I expected that levels of EPP among populations were positively related to breeding density, assessed at the level of the local population.

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SUMMARY

Species often occur in subdivided populations as a consequence of spatial heterogeneity of the habitat. Destruction and fragmentation of habitats due to human land use generally decrease the size and the connectivity of subdivided populations. Due to deterministic and stochastic effects on demographic and genetic parameters, long-term persistence of subdivided populations may be compromised. Theoretical studies have shown that viability of subdivided populations critically depends on the connectivity among local subpopulations, and population models have been developed which predict extinction risks of subpopulations in relation to different levels of connectivity. The lack of empirical data on how subpopulations are spatially organized makes it difficult to judge the general validity of theoretical population models. In fact, predictions derived from population models have rarely been tested with empirical data, although conservation biology focuses on preventing declines and extinctions of populations. However, traditional population definitions are often simplistic, being based for example on the size of a habitat fragment or the geographic clustering of individuals into different breeding units. These population definitions neglect the genetic boundaries of populations if gene flow occurs. The theoretically predicted positive relationship between population size and genetic diversity, which has been supported by many empirical studies, may therefore not be found if gene flow connects populations. Thus, population size may not allow conclusions about the genetic diversity of local populations, which has implications for conservation management of small populations. However, when studying animal behaviour it is useful to focus on populations defined as groups of individuals that have the opportunity to interact in a particular area at a particular time. According to traditional definitions, species living in habitat fragments scattered throughout the landscape occur in geographically distinct local populations and provide the opportunity to test the generality of behaviours by comparing multiple local populations.

An example for a species inhabiting naturally fragmented habitat is the reed bunting (*Emberiza schoeniclus*), a small Palearctic short-distance migratory songbird restricted to wetlands. Wetlands have been destroyed worldwide due to anthropogenic land use (Keddy 1999). Between the 1970s and early 1990s, considerable declines of the reed bunting have been reported for several European countries, for example Germany, Belgium, Finland and England (Heath et al. 2000). Especially in the intensively used agricultural landscapes of south and west Central Europe, habitat destruction has led to extinction of local populations (Glutz von Blotzheim & Bauer 1997) and to reductions in local population sizes (Blümel

1995). The reed bunting is therefore an ideal study species to evaluate the significance of small local populations in population networks and to assess the importance of local population size for the maintenance of genetic diversity in this species. The occurrence of the species in geographically distinct breeding units also provides the opportunity to evaluate the significance of ecological parameters explaining mating-system differences among local populations.

A precondition for the analyses of the spatial distribution of genetic variation in subdivided populations, as well as for the analysis of paternity in avian mating-systems, is the availability of polymorphic molecular markers. In **chapter 1**, I characterized fifteen microsatellite loci specifically designed for the reed bunting. Eleven loci were autosomal and four linked to the Z-chromosome. All loci were characterized and tested in 45 unrelated reed buntings from a Swiss population. Autosomal and z-linked loci were highly polymorphic allowing the inference of spatial genetic structure and the analysis of paternity in reed buntings.

In **chapter 2**, I described the spatial organization of a local system of reed bunting subpopulations. Existing theory proposes three main population models, which predict different levels of connectivity among and extinction risks of subpopulations: patchy population, metapopulation and isolated populations. However, spatially discrete subpopulations are commonly considered to be organized as metapopulations, although explicit tests of metapopulation assumptions are rare. I tested predictions of the three models on the basis of demographic and genetic data, a combined approach so far surprisingly little used in mobile organisms. From 2002-2005, I studied nine subpopulations of the wetland-restricted reed bunting in the southeastern part of the Canton Zurich (Switzerland), from where local declines of the species had been reported. Here, wetlands can be as small as 2.7 ha, often host very few breeding pairs and are separated through intensively used agricultural landscapes. Demographic data referred to dispersal of colour-banded individuals among subpopulations, immigration rates and extinction-/recolonization dynamics. Genetic data were based on the distribution of genetic variability and gene flow among subpopulations derived from the analysis of nine microsatellite loci. Both demographic and genetic data revealed that the patchy population model best described the spatial organization of reed bunting subpopulations. High levels of dispersal among subpopulations, high immigration into the patchy population, and genetic admixture suggested little risk of extinction of both subpopulations and the entire patchy population. This study exemplifies that spatially discrete

subpopulations may be organized in ways other than a metapopulation, which has implications for the conservation of subpopulations and species.

In **chapter 3**, I investigated the importance of local population sizes for the maintenance of genetic diversity in the reed bunting. Reduction of genetic diversity due to genetic drift depends on population effective size, which is often correlated with local population size, i.e. population census or breeding size. However, if gene flow is high, the genetic boundaries of a local population may greatly exceed the geographic area originally used for population delineation. As a consequence, the predicted positive relationship between local population size and genetic diversity may not be found. In reed buntings, the distribution and size of local populations is restricted according to the distribution and size of wetlands. I show that genetic diversity of local reed bunting populations depended on regional abundance of the species rather than on local population sizes, which is in accordance with the high level of gene flow we found among reed bunting populations across Europe. Genetic diversity increased with latitude and was highest in Scandinavia (samples from Norway, located along the presumed centre of the species' distribution range), and lowest in south-central Europe (samples from Switzerland, located along the southern edge of its distribution range). At the species' southern range margin, increased temporal variability in population sizes may have reduced population long-term effective sizes and thus genetic diversity. A further reduction of genetic diversity may be counterbalanced if conservation efforts focus on the protection of remaining wetlands, enhancing habitat quality and thus ultimately population sizes.

In **chapter 4**, I studied the relation between population density and variation of extra-pair paternity (EPP) in reed buntings. Density has been suggested to affect variation of EPP within avian species, because it increases encounter rates and mating opportunities when individuals search for potential extra-pair mates. So far, the significance of density affecting EPP variation in intraspecific comparisons is controversial. However, the absence of a relationship between density and EPP in within- and among-population comparisons as revealed by many empirical studies may mostly be attributed to potentially confounding factors and poor study design. Density measures may not always reflect extra-pair mating opportunities, mate guarding efforts may vary with density, different migration distances and climatic conditions could cause population differences in EPP, and low variation in density and small sample sizes weaken the test power. Taking all those factors into account, I tested if EPP rates within and among local reed bunting populations were related to density. My analyses were based on data from 18 local populations studied over four years. Within populations, the proportion of extra-pair young (EPY) in broods was positively related to

local breeding density. Similarly, among local populations, proportion of EPY was positively associated with population density. I also show that EPP was absent where populations consisted of a single breeding pair, i.e. when no extra-pair mating opportunities were available. My study confirms that density is an important biological factor, which significantly influences the amount of EPP within and among populations, but also supports the view that additional mechanisms contribute to EPP variation.

ZUSAMMENFASSUNG

Als Konsequenz aus der räumlichen Heterogenität ihres Habitats kommen Arten oft in fragmentierten Populationen vor. Zerstörung und Fragmentation des Habitats durch anthropogene Nutzung verringern nicht nur die Grösse, sondern auch die Vernetzung fragmentierter Populationen. Das Langzeitüberleben fragmentierter Populationen ist aufgrund deterministischer und stochastischer Effekte, die Demographie und Genetik fragmentierter Populationen stark beeinflussen können, möglicherweise vermindert. Theoretische Studien haben gezeigt, dass das Langzeitüberleben fragmentierter Populationen stark von der Vernetzung lokaler Subpopulationen abhängt, und es wurden Populationsmodelle entwickelt, die die Vernetzung und das Aussterberisiko solcher Subpopulationen vorhersagen. Die generelle Aussagekraft theoretischer Populationsmodelle lässt sich aber nur schwer feststellen, da wenig empirische Daten zur Verfügung stehen. Die Voraussagen theoretischer Populationsmodelle wurden bisher auch nur selten getestet, obwohl ein ganzer Forschungsbereich, die Naturschutzbiologie, einen ihrer Schwerpunkte darin sieht Bestandsabnahmen und das Aussterben von Populationen zu verhindern. Traditionelle Definitionen einer Population sind in der Regel vereinfachend, da sie beispielsweise Individuen aufgrund geographischer Gegebenheiten in verschiedene Gruppen aufteilen. Diese traditionellen Populationsdefinitionen beachten dabei nicht, dass Populationen über Genfluss miteinander in Verbindung stehen können. Die Grösse einer Population wird häufig als Mass dafür genommen, ob eine Population ausreichend genetisch divers ist, um langfristig überleben zu können. Für durch Genfluss vernetzte Populationen hätte die theoretische Vorhersage, dass die genetische Diversität einer Population von ihrer Grösse abhängt, keine Gültigkeit mehr, woraus sich Folgerungen für den Schutz kleiner Populationen ableiten lassen. In Abhängigkeit vom biologischen Kontext einer Studie oder Fragestellung können traditionelle Populationsdefinitionen aber durchaus sinnvoll sein. Wenn zum Beispiel das Verhalten von Tieren untersucht wird, ist es sinnvoll eine Population so zu definieren, dass sie die Interaktionen von Individuen an einem bestimmten Ort und zu einer bestimmten Zeit umfasst. Ausgehend von traditionellen Populationsdefinitionen kommen Arten, die in fragmentierten Landschaften leben, in geographisch distinkten lokalen Populationen vor. Dies bietet die Möglichkeit Verhalten in verschiedenen Populationen zu untersuchen und zwischen verschiedenen Populationen zu vergleichen.

Ein Beispiel für eine in natürlich fragmentiertem Habitat vorkommende Art ist die Rohrammer (*Emberiza schoeniclus*). Diese paläarktisch verbreitete Singvogelart ist ein

Kurzstreckenzieher dessen Vorkommen sich auf Feuchtgebiete beschränkt. Feuchtgebiete wurden und werden durch anthropogene Nutzung weltweit zerstört. Aufgrund dessen nahm zwischen den Siebziger und Neunziger Jahren des letzten Jahrhunderts der Rohrammerbestand in verschiedenen europäischen Ländern wie Deutschland, Belgien, Finnland oder England ab. Besonders in den südlichen und westlichen, landwirtschaftlich intensiv genutzten Teilen Mitteleuropas hat die Zerstörung der Feuchtgebiete zu Bestandsabnahmen und zum Aussterben lokaler Rohrammerpopulationen geführt. Aufgrund ihrer Habitatspezifität ist die Rohrammer eine ideale Art, um die Bedeutung kleiner Populationen in Populationsnetzwerken sowie die Bedeutung von Populationsgrössen für den Erhalt genetischer Diversität zu untersuchen. Dass die Rohrammer in geographisch distinkten Gruppen brütet, ermöglicht es ausserdem den Einfluss ökologischer Parameter auf Aspekte des Paarungssystems der Rohrammer zwischen verschiedenen geographisch distinkten Populationen zu vergleichen.

Eine Voraussetzung für derartige Analysen ist die Verwendung polymorpher molekularer Marker. In **Kapitel 1** charakterisiere ich fünfzehn Mikrosatellitenloci, die spezifisch für die Rohrammer entwickelt wurden. Elf dieser Loci waren autosomal, die restlichen vier geschlechtsspezifisch (d.h. sie lagen auf dem Z-Chromosom). Alle Loci wurden in 45 nicht miteinander verwandten Rohrammern einer schweizer Population charakterisiert und getestet. Sowohl die autosomalen als auch die z-gelinkten Loci waren hochpolymorph, was die Untersuchung der räumlichen genetischen Struktur von Rohrammerpopulationen und von Vaterschaftsanalysen möglich machte.

In **Kapitel 2** beschreibe ich die räumliche Organisation eines lokalen Systems von Rohrammersubpopulationen. Aus der Theorie sind drei Populationsmodelle bekannt, die Voraussagen über Vernetzung und Aussterbeereignisse lokaler Subpopulationen machen. Herkömmlicherweise nimmt man für geographisch diskrete Subpopulationen eine Metapopulationsstruktur an. Tests, die überprüfen, ob die räumliche Organisation der Subpopulationen die Annahmen einer Metapopulation auch erfüllt, sind allerdings selten. Ich testete die Voraussagen dreier Populationsmodelle auf der Grundlage demographischer und genetischer Daten. Dieser kombinierte Ansatz wurde bis jetzt erstaunlich selten gewählt, zumindest für mobile Organismen. Zwischen 2002 und 2005 untersuchte ich neun Subpopulationen der Rohrammer im südöstlichen Teil des Kantons Zürich (Schweiz), von wo vereinzelt Bestandsrückgänge der Art beschrieben wurden. In diesem Teil des schweizerischen Mittellandes sind Feuchtgebiete teilweise nur 2.7 ha gross und durch landwirtschaftlich intensiv genutzte Flächen voneinander getrennt. Farbberingte Individuen lieferten demographischen Daten über die Dispersion zwischen Subpopulationen, Immigrationsraten

und Aussterbe- und Wiederbesiedlungsereignissen. Die genetischen Daten basierten auf der molekularen Information von neun Mikrosatellitenloci und beschreiben die Verteilung genetischer Variation sowie den Genflusses zwischen den Subpopulationen. Sowohl die demographischen als auch die genetischen Daten zeigen, dass die Organisation der Subpopulation am besten durch das Modell der 'Patchy population' beschrieben wird. Das hohe Mass an Dispersal zwischen den Subpopulationen, die hohe Immigrationsrate in die gesamte 'Patchy population' und die fehlende genetische Differenzierung zeigen, dass sowohl für die einzelnen Subpopulationen als auch für die gesamt 'Patchy population' nur eine geringe Aussterbewahrscheinlichkeit besteht. Diese Studie ist ein Beispiel dafür, dass geographisch diskrete Subpopulationen räumlich durchaus anders als Metapopulationen organisiert sein können, woraus sich bestimmte Folgerungen für den Schutz von Subpopulationen und Arten ableiten lassen.

In **Kapitel 3** untersuchte ich die Bedeutung lokaler Populationsgrössen für den Erhalt genetischer Diversität am Beispiel der Rohrammer. Die Verringerung genetischer Diversität durch genetische Drift hängt generell von der effektiven Populationsgrösse ab, die oft mit der lokalen Populationsgrösse korreliert ist. Lokale Populationsgrössen werden meist als die Anzahl der lokalen Individuen oder als die Anzahl brütender Individuen angegeben. Wenn Genfluss zwischen lokalen Populationen hoch ist, kann die genetische Abgrenzung einer lokalen Population allerdings den ursprünglich für die Populationsdefinition benutzten lokalen geographischen Rahmen bei weitem überschreiten. Der theoretische vorhergesagte Zusammenhang zwischen lokaler Populationsgrösse und dem Ausmass genetischer Diversität kann dadurch möglicherweise nicht mehr gefunden werden. Bei der Rohrammer sind lokale Populationsgrössen von der Verteilung und der Grösse von Feuchtgebieten abhängig. In dieser Studie zeige ich, dass aufgrund hohen Genflusses die genetische Diversität lokaler Rohrammerpopulationen von der regionalen Dichte der Rohrammer und nicht von den lokalen Populationsgrössen abhängt. Mit zunehmendem Breitengrad nahm die genetische Diversität zu und war am höchsten in Skandinavien (Norwegen) und am niedrigsten am südlichen Rand des Rohrammerverbreitungsgebiets (Schweiz). Temporäre Veränderungen der lokalen Populationsgrössen über längere Zeiträume hinweg könnten dazu geführt haben, dass die durchschnittlichen effektiven Populationsgrössen und damit auch die genetische Diversität am südlichen Rand des Verbreitungsgebiets abnahmen. Der weiteren Abnahme genetischer Diversität könnte allerdings durch geeignete Schutzmassnahmen entgegengewirkt werden, wenn diese Massnahmen eine Qualitätsverbesserung des Rohrammerhabitats und damit letztlich auch eine Vergrösserung der lokalen Populationen zur Folge haben.

In **Kapitel 4** untersuchte ich den Einfluss der Brutdichte auf die Häufigkeit von Fremdvaterschaften bei der Rohrammer. Theoretisch gesehen führt zunehmende Dichte einerseits dazu, dass sich Männchen und Weibchen ausserhalb des sozialen Paarbundes häufiger begegnen. Andererseits ergeben sich mit zunehmender Dichte generell mehr Möglichkeiten einen potentiellen Partner für das Fremdgehen zu finden. Allerdings hat sich bisher die Bedeutung der Dichte als Erklärung für die hohe Variation im Ausmass von Fremdvaterschaften zwischen verschiedenen Populationen derselben Art nicht als sehr überzeugend herausgestellt. Dass viele Studien keinen Zusammenhang zwischen Dichte und der Häufigkeit von Fremdvaterschaften gefunden haben ist möglicherweise eine Folge ihres methodischen Aufbaus, beziehungsweise der Nichtberücksichtigung von Faktoren, die das Untersuchungsergebnis beeinträchtigt haben könnten. Die in diesen Studien verwendeten Masse für Dichte spiegelten nicht immer die Möglichkeit des Fremdgehens wider. Unterschiedliche Migrationsdistanzen und klimatische Verhältnisse zwischen Populationen könnten ebenfalls Ergebnisse verfälscht haben. Hinzu kommt, dass sowohl eine zu geringe Bandbreite an Dichten, unter denen die Häufigkeit von Fremdvaterschaften gemessen wurde, als auch eine zu geringe Stichprobengrösse die Aussagekraft der verwendeten Tests verringert haben könnten. Unter Berücksichtigung all dieser Faktoren testete ich, ob es einen Zusammenhang zwischen der Häufigkeit von Fremdvaterschaften innerhalb und zwischen verschiedenen lokalen Rohrammerpopulationen und der jeweiligen Dichte gab. Meine Analysen basierten auf Daten von 18 lokalen Populationen, die während vier Jahren untersucht wurden. Innerhalb der Populationen war die Häufigkeit von Fremdvaterschaften positiv mit der lokalen Brutdichte korreliert. Beim Vergleich zwischen verschiedenen Populationen waren Häufigkeiten von Fremdvaterschaften positiv mit der Populationsdichte korreliert. In lokalen Populationen, die nur aus einem einzigen Brutpaar bestanden und in denen es somit keine Möglichkeit des Fremdgehens gab, kam es auch nie zu Fremd-vaterschaften. Damit bestätige ich in meiner Studie die Hypothese, dass Dichte tatsächlich ein biologisch relevanter Faktor ist, der einen signifikanten Einfluss auf die Häufigkeit von Fremdvaterschaften innerhalb und zwischen verschiedenen Populationen hat. Darüber hinaus bestätigen meine Ergebnisse die Ansicht, dass neben der Dichte weitere Mechanismen die Häufigkeit von Fremdvaterschaften beeinflussen.

CHAPTER 1

Isolation, characterization and multiplex genotyping of eleven autosomal and four sex-linked microsatellite loci in the reed bunting *Emberiza schoeniclus* (Emberizidae, Aves)

Christian Mayer, Karin Schiegg, Gilberto Pasinelli

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Abstract

Fifteen highly polymorphic microsatellite loci were characterized in the reed bunting *Emberiza schoeniclus*. Eleven loci were autosomal and four linked to the Z-chromosome. All loci were characterized and tested in 45 unrelated reed buntings from a Swiss population. Autosomal loci displayed 7-17 and sex-linked loci 4-13 alleles with heterozygosities ranging from 0.756-0.933 and 0.478-0.957, respectively. These loci will be used in population genetic and mating system studies of reed buntings.

Introduction

The reed bunting (*Emberiza schoeniclus*) is a small dimorphic migratory passerine widely distributed in Europe and Asia. Between the 1970s and 1990s declines in population sizes by 20-50 % have been reported for several European countries (Heath *et al.* 2000) for example Germany, Italy, Belgium, Finland and England. The population decline, presumably resulted from drainage and loss of wetlands, raises an interest for population genetics of the species. Moreover, although socially monogamous, reed buntings have high rates of extra pair paternity (Dixon *et al.* 1994), which can only be assessed with molecular markers. We therefore characterized a set of polymorphic dinucleotide microsatellite loci for the reed bunting.

Material and Methods

Blood samples were collected from 10 reed buntings (five males and five females) descending from populations of the Zürcher Oberland (Canton Zürich, Switzerland). Genomic DNA was isolated from whole blood using a standard phenol-chloroform extraction protocol (Sambrook *et al.* 1989). An enriched library was made by ECOGENICS GmbH (Zurich, Switzerland) from size selected genomic DNA ligated into TSPAD-linker (Tenzer *et al.* 1999) and enriched by magnetic bead selection with biotin-labelled $(CA)_{13}$ and $(GA)_{13}$ oligonucleotide repeats (Gautschi *et al.* 2000a, b). Of 384 recombinant colonies screened, 130 gave a positive signal after hybridization. Plasmids from 96 positive clones were sequenced and primers were designed for 21 microsatellite inserts. Of these 18 were tested for polymorphism.

To determine optimal amplification conditions, a PCR with unlabeled primers was performed in a 10 µl reaction volume including 1 x PCR buffer (Qiagen, containing Tris-Cl, KCl, $(NH_4)_2SO_4$ and a final concentration of 150 µM $MgCl_2$), 0.3 µM of each forward and reverse primer, 0.5 U Hotstar*Taq* (Qiagen) and 1 ng DNA. The best amplification conditions were as follows: 95 °C for 15 min followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and a final elongation step of 72 °C for 10 min. The amplified products were separated on precast Spreadex® EL400 and EL600-Gels on a SEA 2000^{TM} advanced submerged gel electrophoresis apparatus (Elchrom Scientific AG, Switzerland). Products were visualized using SYBR Gold (Molecular Probes) and scored against the M3 Marker (Elchrom Scientific AG, Switzerland). Of the 18 loci, 17 were found to be polymorphic.
To evaluate primer performance and to assess variation among individuals 45 reed bunting samples (23 males and 22 females) from Lake Greifensee in the Zürcher Oberland (Canton

Zürich, Switzerland) were genotyped for 15 loci by setting up two multiplex PCRs with eight and seven loci (Table 1). The Qiagen® Multiplex Kit was used following exactly the protocol except for changes in the optimal annealing temperature and primer concentrations (Table 1). The fluorescence labelled amplified fragments were visualized on an ABI 3700 Avant capillary sequencer and allele sizes were determined in relation to an internal size standard (GeneScan-500 LIZ) using GENEMAPPER Vers.3.7.

Results

All loci were polymorphic with the number of alleles ranging from four to 17 (Table 1). Four loci were found to be z-linked with all females being hemizygous (Table 1). None of the loci showed significant evidence for the presence of null alleles, when analysed with MICRO-CHECKER 2.2.3 (van Oosterhout *et al.* 2004). ARLEQUIN 3.1 (Excoffier *et al.* 2005) was used to estimate observed and expected heterozygosities (Table 1) and a Markov chain method implemented in GENEPOP on the web (http://genepop.curtin.edu.au/) (Raymond & Rousset 1995) was used to determine, whether the loci were in Hardy-Weinberg equilibrium and in linkage disequilibrium. Except for locus Emb116 with a heterozygote deficit, and locus Emb117, showing a heterozygote excess, all loci were in Hardy-Weinberg equilibrium (Table 1). None of the loci was in linkage disequilibrium after Bonferroni correction. These microsatellite loci will be used to examine genetic variability and to assess population genetic structure of reed bunting populations in Austria, Germany, The Netherlands, Norway, Poland and Switzerland. Furthermore, they will be used for paternity analysis and the sex-linked loci provide a useful tool to study sex-biased dispersal.

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Table 1 Characterization and variability of 15 loci isolated from *Emberiza schoeniclus*. The optimal annealing temperature (T_a) for each of the two multiplex PCR reactions was 58°C. Number of successfully genotyped indiviuals (n_s), size range, number of alleles, observed (H_o) and Nei's expected (H_e) heterozygosity across 45 samples analysed. Significant deviations from Hardy-Weinberg expectations are indicated by an asterisk ($p < 0.05$). For sex-linked loci (indicated by a z) values were calculated for males only (n = 23).

PCR	Locus	GenBank Acc. no.	Primer sequence (5' – 3')	Repeat motif	Primer (µM)	Dye	n_s	Size range	Allele no.	H_o	H_e
PCR 1	Emb89	EF601685	F: GCACCACTTGTTCTTCTTGG	$(CT)_9$ CG $(CT)_{16}$	0.26		44	81-125	10	0.756	0.804
		EF601685	R: CAAGAAAACTTGCAATGAAAATG		0.26	6-FAM					
	Emb27	EF601686	F: TCCCCATGATGGTCTGTACC	$(GT)_{18}$	0.04	VIC	45	97-145	13	0.822	0.862
		EF601686	R: GCTGACTGCTTGGCTGGAC		0.04						
	Emb107[z]	EF601687	F: CTGTGTAATGTAAGTTTGGCCCTTAC	$(GT)_{12}$	0.24	NED	43	157-169	4	0.478	0.434
		EF601687	R: CTGTGCAGCAGATCAATCACTATG		0.24						
	Emb19	EF601688	F: CTGCAGATTTCAGGAGGTTG	$(GT)_{18}$	0.24	PET	44	134-178	13	0.844	0.869
		EF601688	R: GCATGGTAATCGTGGTGTTG		0.24						
	Emb12	EF601689	F: ATCTGTAAGGAGAGCATGAAATAAC	$(GT)_{22}$	0.2		45	168-194	13	0.889	0.865
		EF601689	R: TAGGAACTGGCTGGGACATC		0.2	6-FAM					
	Emb90	EF601690	F: TGCTCACTCTTGTCCTGTGC	$(CT)_{11}$ CG $(CT)_{10}$	0.13		45	175-205	12	0.867	0.854
		EF601690	R: TCCGTGAAAACCTACCTAAGAAC		0.13	VIC					
	Emb79[z]	EF601691	F: TGAGGTTCTAGGTAGCGTGATG	$(GT)_{17}$	0.28	NED	43	205-225	7	0.913	0.811
		EF601691	R: ATCTGAGCTGCAATCTCTTTCG		0.28						
	Emb03	EF601692	F: GCAGGTATTAGAGCCCCTTGTC	$(GT)_{17}$	0.49	PET	44	209-247	17	0.867	0.907
		EF601692	R: GGGCTGTCTGGGATTTTTATTC		0.49						
PCR 2	Emb17	EF601693	F: GCGCTTGCTATGAGCAGATG	$(GT)_{17}$ AT $(GT)_5$	0.36	PET	45	105-133	7	0.756	0.755
		EF601693	R: CACCCTATTATGTAAAGAGGATGC		0.36						

Emb116	EF601694	F: CCAAAAGCAAAACCTGGATG	$(CA)_{18}$ GACAGA	0.01		45	130-158	11	0.778	0.855*
	EF601694	R: TCCCCTTACACAGAGAAATATGC	$(CA)_5$	0.01	VIC					
Emb112	EF601695	F: GTGAAGAGGTGCCTTTTAGGAC	$(GT)_6$ AT $(GT)_{15}$	0.2	6-FAM	45	128-182	15	0.933	0.904
	EF601695	R: CGGGGAAGGAAAAGCACT		0.2						
Emb81	EF601696	F: GAGAGACTGAGGGCATTTTGAG	$(CT)_4$CCCTCC	0.12	PET	45	150-202	15	0.889	0.910
	EF601696	R: TGCAGCACTCAAGAGATAGGAGT	$(CT)_4$CCCTCC $(CT)_{14}$	0.12						
Emb84 [z]	EF601697	F: TGCCTTGTGCAGTTCTCATC	$(GA)_{26}$ … $(GA)_3$ …	0.5	NED	36	161-219	13	0.957	0.917
	EF601697	R: AGAGAAGTGGCCAAGCTCAG	$(GA)_6$ …$(GA)_6$	0.5						
Emb07	EF601698	F: ATCAGCCAGTTTGGGGAAC	$(GT)_{20}$	0.4	VIC	45	160-194	12	0.867	0.83
	EF601698	R: TCTTTTTCAGACCTCAGGTAAGC		0.4						
Emb117	EF601699	F: GAACTCCTGAGCTTTGATCCAG	$(GT)_{21}$ GC $(GT)_5$	0.12		44	212-245	6	0.783	0.715*
[z]	EF601699	R: GGCTGGCTTGCTTATGGTG	GGGTGG $(GT)_4$	0.12	6-FAM					

CHAPTER 2

Patchy population structure in a short-distance migrant: evidence from genetic and demographic data

Christian Mayer, Karin Schiegg, Gilberto Pasinelli

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Abstract

Species often occur in subdivided populations as a consequence of spatial heterogeneity of the habitat. To describe the spatial organization of subpopulations, existing theory proposes three main population models, which predict different levels of connectivity among and extinction risks of subpopulations: patchy population, metapopulation and isolated populations. However, spatially discrete subpopulations are commonly considered to be organized as metapopulations, although explicit tests of metapopulation assumptions are rare. Here, we test predictions of the three models on the basis of demographic and genetic data, a combined approach so far surprisingly little used in mobile organisms. From 2002-2005, we studied nine subpopulations of the wetland-restricted reed bunting (*Emberiza schoeniclus*) in the southeastern part of the Canton Zurich (Switzerland), from where local declines of the species have been reported. Here, wetlands are as small as 2.7 ha and separated through intensively used agricultural landscapes. Demographic data referred to dispersal of colour-banded individuals among subpopulations, immigration rates and extinction-/recolonization dynamics. Genetic data were based on the distribution of genetic variability and gene flow among subpopulations derived from the analysis of nine microsatellite loci. Both demographic and genetic data revealed that the patchy population model best described the spatial organization of reed bunting subpopulations. High levels of dispersal among subpopulations, high immigration into the patchy population, and genetic admixture suggested little risk of extinction of both subpopulations and the entire patchy population. This study exemplifies that spatially discrete subpopulations may be organized in ways other than a metapopulation, which has implications for the conservation of subpopulations and species.

Introduction

Species often occur in subdivided local populations as a consequence of spatial heterogeneity of the habitat. Heterogeneity of the habitat may have two reasons (Fischer & Lindenmayer 2007). First, the habitat has a naturally fragmented distribution or second, formerly continuous habitats have been destroyed and subdivided into fragments due to anthropogenic landscape modifications. Because the ongoing destruction and fragmentation of habitats due to human land use generally reduces the size and the connectivity of the remaining local populations, species having evolved both in continuous habitats and in naturally fragmented habitats may be affected (Frankham *et al.* 2002). However, the consequences of continued habitat loss and fragmentation for species occurring in naturally fragmented habitats are little studied.

Small local population size and restricted gene flow lead to genetic differentiation between populations. Population subdivision intensifies the effects of genetic stochasticity in local populations leading to loss of genetic variation through random genetic drift (Frankham *et al.* 2002) and loss of fitness due to inbreeding (Frankham 1995; Keller *et al.* 2002). Loss of genetic variation and fitness are predicted to enhance the risk of local extinction, especially in populations that have recently declined in size. Theoretical studies have shown that viability of populations critically depends on the connectivity among local populations (Gilpin & Hanski 1991; Harrison 1991).

To describe the spatial organization of subpopulations, three main population models can be identified. These models of spatial population structure represent steps of a continuum, here presented in the order of decreasing connectivity: (1) patchy population, (2) metapopulation and (3) isolated populations. Other forms of spatial organizations of subpopulations (mainland-island, source-sink) were not considered because these forms are very similar to each other and to the patchy population or metapopulation scenario. For example, source-sink systems may occur in either metapopulations or patchy populations, making specific predictions for distinguishing source-sink population structure from metapopulation or patchy population structure difficult if not impossible. According to the first model, subpopulations are considered to be part of a patchy population (Harrison 1991). The subpopulations are well connected by dispersal, that is, they represent one single population with little potential for local extinction of single subpopulations. The second model proposes that subpopulations are organized as a metapopulation, here defined as a collection of partially isolated habitat patches, which may support local breeding subpopulations, with extinction and recolonization of subpopulations occurring. Due to

recolonizations of extinct subpopulations the entire metapopulation usually persists much longer than each of the local subpopulations (Levins 1970). In the third model, subpopulations are isolated from each other, that is, the subpopulations are separate small populations. "Subpopulations" may represent fragments of a formerly continuous population. Once extinct, fragments will not be recolonized (Frankham *et al.* 2002).

Distinguishing between these models of spatial population structure by empirical estimates of dispersal among subpopulations is difficult, for example due to the great logistic challenges associated with banding and re-observing individuals in multiple subpopulations. In addition, direct observations of dispersal only partially reveal the patterns of individual movements (Koenig *et al.* 1996) and may represent an inadequate estimation of gene flow, because gene flow requires successful reproduction of the immigrant (Boughton 1998; Hedrick 2005). Furthermore, the significance of direct dispersal estimates is limited to the study of contemporary population dynamics. In turn, indirect estimates of gene flow based on the distribution of allele frequencies among populations depend on levels of gene flow averaged over long times and may therefore be the result of past rather than current dispersal (Slatkin 1987). However, identification of real-time migrants using genetic assignment methods (see Manel *et al.* 2005 for review) is also possible but restricted to cases where populations are genetically sufficiently structured (Cornuet *et al.* 1999; Paetkau *et al.* 2004). The most promising approach is therefore to combine direct and indirect estimates of dispersal and gene flow because they yield information on current and past gene flow (Slatkin 1987). Surprisingly, however, relatively few studies on mobile organisms have adopted such a combined approach so far.

The three models of spatial population structure make contrasting predictions with respect to dispersal and gene flow patterns and the distribution of genetic variation within and among local subpopulations (Table 1). (1) The patchy population model predicts high dispersal among all subpopulations and, due to generally high levels of dispersal, also substantial immigration into the patchy population. Since local extinction risk is low, we expect no extinctions or recolonizations of subpopulations. The patchy population model further predicts no significant genetic differentiation among subpopulations due to the high amount of gene flow homogenizing any genetic structure (Harrison 1991). The entire patchy population is therefore in Hardy-Weinberg equilibrium and isolation-by-distance is not expected to occur on local scales (Slatkin 1993). Linkage disequilibrium among unlinked nuclear loci may be caused by the effects of genetic drift in small populations (Hedrick 2005). However, due to the high amount of gene flow among subpopulations, genetic drift acts at the

level of the entire patchy population, and the magnitude of linkage disequilibrium within single subpopulations should therefore not be related to subpopulation size. Population structure can also be assessed without imposing any preconceived assumptions on the number and distribution of subpopulations. In this case, individuals of the patchy population are expected to form a single genetic cluster. (2) The metapopulation model predicts dispersal to be restricted and thus mostly occurring among neighbouring populations. Accordingly, immigration into the metapopulation is low. Metapopulation structure predicts the occurrence of extinctions and recolonizations of subpopulations (Hanski 1999). All local subpopulations have a substantial probability of extinction but patches that are unsettled due to local extinction events are recolonized by founder individuals from other subpopulations. The metapopulation model further predicts that gene flow among subpopulations is low and that subpopulations are hence genetically significantly differentiated (Hastings & Harrison 1994). Analysis at total population level, i.e. when considering all subpopulations collectively, should reveal significant deviation from Hardy-Weinberg equilibrium with a deficit of heterozygotes, since the whole metapopulation is composed of genetically differing subpopulations (Wahlund 1928, as cited in Hedrick 2005). Since genetic exchange occurs mainly between neighbouring subpopulations, genetic differentiation is explained by isolation-by-distance (Slatkin 1993). Depending on subpopulation size, stochastic fluctuations result in nonrandom associations between alleles at different loci (Hedrick 2005). The strength of linkage disequilibrium in subpopulations of the metapopulation should therefore be negatively related to subpopulation size. The metapopulation is expected to consist of more than one genetic cluster. (3) The isolated population model predicts no dispersal among and no immigration into "subpopulations" (Table 1). Isolated populations may go extinct but will not be recolonized. Due to isolation, subpopulations are highly differentiated from each other. Differences in allele frequencies among subpopulations cause deviation from Hardy-Weinberg equilibrium at total population level, resulting in heterozygote deficiency (Wahlund 1928, as cited in Hedrick 2005). Since subpopulations evolve independently due to complete isolation, genetic differences between subpopulations are not related to geographic distance (Hutchison & Templeton 1999). Within subpopulations, the strong effects of genetic drift and inbreeding generate a relatively high level of linkage disequilibrium, which should be negatively related to subpopulation size (Hedrick 2005). Due to complete isolation, the number of genetic clusters is expected to correspond to the number of subpopulations.

In this study we investigate the spatial population structure of subpopulations of the reed bunting (*Emberiza schoeniclus*). The reed bunting is a small Palaearctic short-distance

migratory songbird restricted to wetlands. Due to its specific habitat requirements, the spatial distribution and size of reed bunting subpopulations depend on the distribution and size of wetlands (Glutz von Blotzheim & Bauer 1997). Wetlands have been destroyed worldwide due to anthropogenic land use (Keddy 1999). As a consequence, the reed bunting has strongly suffered from the destruction and perhaps also the deterioration of its habitat (Blümel 1995). Between the 1970s and early 1990s, reed bunting declines have been reported for several European countries, for example in Italy, Germany, Belgium, Finland and England (Heath *et al.* 2000).

In Switzerland, wetland protection programmes were developed in the 1970s to stop further destruction of wetland habitat. Since 1975 the size of wetlands in the Swiss lowlands has remained constant (Weggler *et al.* 2004). Accordingly, the overall distribution of reed buntings in Switzerland has not changed in the recent years, except that the species has disappeared at the edges of its distribution range (Antoniazza 1998). In the southeastern part of the Canton Zurich, where this study took place, wetlands are as small as 2.7 ha and separated by intensively managed agricultural landscapes. Between 1993 and 2006, a decline of breeding reed buntings has been reported for the three largest wetlands in the Canton Zurich for reasons yet unknown (Weggler & Widmer 2001; M. Weggler, personal communication). Reed buntings regularly breed in these wetlands, but it is unknown, whether and how the wetlands are connected by dispersal, given the apparently short dispersal distances reported for this species (Paradis *et al.* 1998).

To summarise, the specific objectives of this paper are to assess the spatial organisation of reed bunting subpopulations by testing theoretical predictions of the three population models with demographic and genetic data. According to the best-supported model, we discuss implications for the conservation management of this locally declining species.

Material and Methods

Sampling

The study took place in a 200 km^2 area of the Zürcher Oberland in the south eastern part of the Canton Zurich, Switzerland. Here, 19 wetland fragments were examined from 2002 to 2005 in the context of a study on the population ecology of the reed bunting (see Pasinelli *et al.* (2008a) for further details). All these fragments are nature reserves offering, to variable extent, old reed habitats, which represent the only suitable breeding habitat for reed buntings in the whole study area. The presence of old reed *Phragmites* sp. is the most

important cue for territory establishment when males return from the wintering grounds (Surmacki 2004). To prevent the overgrowth with reed all fragments are partially mown in autumn. Only small bands of reed along water bodies as well as a few distinct patches are spared from the annual cut. All fragments border at intensively used agricultural areas. Because some of these fragments clearly hosted too few birds to for reliable estimates of genetic parameters we aggregated fragments to nine subpopulations according to their spatial location (Fig. 1). Within subpopulations, distances between pooled fragments were below 900 m, which has been reported to be the average juvenile dispersal distance in the reed bunting (Paradis *et al.* 1998). Because pooling of fragments may have an impact on the distribution of genetic variation of subpopulations, we first tested for genetic differentiation among fragments, which were to be pooled using the same methods as described later in the 'Genetic data' section. No genetic differences and also no deviation from Hardy-Weinberg equilibrium could be detected. We found a deviation from Hardy-Weinberg equilibrium in the subpopulation Lützelsee (Table 3). However, this deviation was not due to the pooling of three fragments, because deviation from Hardy-Weinberg equilibrium was also detected, when the fragment Lützelsee was separately analysed. Subpopulations hosted 1 to 50 breeding pairs per year. All breeding pairs were completely monitored in each subpopulation except in the three largest subpopulations Greifensee, Lützelsee, and Pfäffikersee, where we focused on a sub-sample of at least 10 breeding pairs annually (Table 2).

Reed buntings were annually monitored from early March, when males return from their wintering grounds, to the end of the breeding period in early August. Nests were located by observing females building their nest, leaving their nest and returning to it during incubation, or when the parents were feeding the young. The young were banded between nestling day 6 and 9, with each nestling obtaining a numbered aluminium ring as well as a unique combination of three coloured plastic rings allowing individual identification by telescope observation. To catch and band males, a mist net was set up at the border of a territory, and a loudspeaker was placed in front of the net. Females were caught with a net placed at a distance of at least 2 m from the nest, when flying to the nest to feed their young. Adults were individually marked (see above) and a blood sample (max. 100 µl) from each individual was taken by puncturing the brachial vein (permission number from the Cantonal Veterinary Office Zurich: 169/2001). Blood was absorbed with heparinized microcapillaries. Samples were either stored in microcapillaries directly or blown into APS-buffer (Arctander 1988) and stored at -20° C. A total of 253 breeding individuals (132 males and 121 females) were used for data analysis.

Demographic data

To get data on dispersal and immigration we annually monitored all reed buntings of all subpopulations at least twice a week from early March to early August and documented their movements within and among all subpopulations. From May to July 2003 – 2007 we also systematically searched for banded birds outside the intensively monitored old reed areas of the three largest wetland fragments and opportunistically in wetlands of the Canton Zurich outside the 200 km^2 – study area. We focused our search for banded reed buntings on wetlands, because the species does not use habitats other than wetlands during breeding time in the Canton Zurich. The period between May and July corresponds to the breeding season of the reed bunting in our study area; individuals observed during that time are considered territorial breeding birds. Non-breeding territorial individuals were extremely rare (unpublished data, G. Pasinelli). We considered as natal dispersal event the movement of a bird born in a subpopulation and breeding in a different subpopulation or outside the study area in the subsequent year, respectively. Breeding dispersal was defined as the movement of an adult individual to another subpopulation or to outside the study area between two breeding attempts, either within the same or between subsequent years.

Obtaining estimates of immigration rates was complicated because not all individuals in each subpopulation could be banded every year (proportion of banded individuals across years: mean ± SD, 83.73 % ± 4.77). We calculated annual immigration rate over all subpopulations by dividing the number of individuals that were unbanded and territorial at the beginning of the breeding season by the entire number of individuals (banded and unbanded). Unbanded individuals may cause problems in estimating immigration rate because, for a specific year, it is not possible to distinguish between new unbanded individuals (i.e. true immigrants) and unbanded philopatric individuals that already have bred in the study area in the previous year. To account for the potential bias, we treated unbanded individuals in the calculation of immigration rate in three different ways, in the following exemplified for 2003: (1) we assumed that all individuals who remained unbanded at the end of the breeding season in 2002 did not return to the study area in 2003. All unbanded individuals in 2003 are therefore treated as true immigrants, which will give the maximum immigration rate; (2) we assumed that all these individuals were philopatric, which will give the minimum immigration rate. The true immigration rate will lie between the minimum and maximum estimates, and we further approximated it by treating unbanded individuals of the previous year as immigrants, but correcting for the number of assumed unbanded philopatric individuals. This was done in the following way: (3) assuming that the probability of being philopatric is the

same for both unbanded and banded breeding individuals (philopatry rates of banded adult females across years: 0.436 (95% CI 0.428-0.445), Pasinelli *et al.* 2008b, submitted) we determined the number of unbanded but presumably philopatric individuals for 2002. This number was then substracted from the number of unbanded individuals observed in 2003.

A subpopulation was defined as extinct, when no territorial bird settled despite the presence of suitable habitat. Recolonizations were considered to have occurred, when unoccupied suitable habitat was subsequently held by breeding individuals.

Genetic data

Laboratory analysis

For DNA preparation two extraction kits were used: the Roche "High Pure PCR Template Preparation Kit" following exactly the protocol (Vogelstein & Gillespie 1979) and the Qiagen "Biosprint 96 DNA Blood Kit". We used a set of 11 autosomal microsatellite loci (Emb 03, Emb 07, Emb 12, Emb 17, Emb 19, Emb 27, Emb 79, Emb 81, Emb 89, Emb 90, Emb 112, and Emb 116) for population genetic analysis as described previously (Mayer *et al.* 2007). Polymerase chain reaction amplification and genotyping were conducted as described in (Mayer *et al.* 2007).

Genetic variation

Genetic variation within each subpopulation was measured by using allele frequency data, from which the number of alleles per locus (A), the allelic richness (R) averaged over loci (Petit *et al.* 1998) and F_{IS} were calculated with FSTAT 2.93 (Goudet 2001). Observed (H_o) and expected (H_e) heterozygosities (Nei 1987) were calculated with ARLEQUIN 3.1 (Excoffier *et al.* 2005). Departure from Hardy-Weinberg expectations was tested with GENEPOP on the web (http://genepop.curtin.edu.au/) (Raymond & Rousset 1995), both within each subpopulation and at total population level, i.e. when subpopulations were pooled. These tests were conducted using a Markov chain with 5’000 batches each iterated 1’000 times and a dememorization number of 10000 (Guo & Thompson 1992; Raymond & Rousset 1995).

Null alleles

The software MICRO-CHECKER 2.2.3 (van Oosterhout *et al.* 2004) was used to check for appearance of long allele dropout or scoring errors due to stutter rate and to test for the occurrence of null alleles. We found evidence for the existence of null alleles at loci Emb

81 and Emb 116. Conducting statistical analysis on data sets containing null alleles may lead to misinterpretation of the data and wrong biological conclusions (Dewoody *et al.* 2006). Since these two loci caused significant deviation from Hardy-Weinberg equilibrium, we excluded them from all analyses.

Linkage disequilibrium

The strength of linkage disequilibrium within each subpopulation was assessed using r_d (Agapow & Burt 2001) as a measure of multilocus linkage disequilibrium. This measure is equal to the index of association (I_A) but corrected for the number of loci used for analysis. We also used a randomization procedure (1000 iterations) to test the hypothesis of complete panmixia between alleles at different loci. Calculations were done in MULTILOCUS 1.3 (Agapow & Burt 2001), and r_d – values were then regressed on subpopulation size using SPSS 12.0.2 for Windows.

Genetic differentiation

We tested for allele frequency differences between subpopulations with an exact probability test using GENEPOP on the web (http://genepop.curtin.edu.au/) (Raymond & Rousset 1995). Differentiation among subpopulations was described with F_{ST} (Wright 1951). We did not use R_{ST} (Chakraborty & Nei 1982; Slatkin 1995), because the allelic distributions of five microsatellite loci revealed large gaps and one locus showed single base pair shifts, suggesting that these loci did not follow a strict stepwise mutation model. In addition, F_{ST} has been shown to be the best estimator in cases, when genetic differentiation among populations is expected to be low (Balloux & Goudet 2002). F_{ST} estimates were calculated over all subpopulations and for all subpopulation pairs according to Weir & Cockerham (1984) and tested for significance by permuting genotypes among samples (5000 permutations) with FSTAT 2.9.3.2 (Goudet 2001). To assess whether geographical distance between subpopulations may explain genetic differentiation, isolation by distance was examined using a Mantel test (90'000 permutations) in ARLEQUIN 3.1 (Excoffier *et al.* 2005). Pairwise genetic distance defined as $F_{ST}/(1-F_{ST})$ was regressed on the logarithms of geographical distance. This regression is considered linear in a two-dimensional model (Rousset 1997).

Differentiation among subpopulations was also evaluated with the model-based clustering method of Pritchard *et al.* (2000) implemented in STRUCTURE 2.1. This method uses a Bayesian approach to detect potentially existing genetic structure without imposing any preconceived ideas of population substructure. The method assigns individuals, based on

Hardy-Weinberg expectations, to a user-defined number of genetic clusters (*K*). A Markov chain Monte Carlo (MCMC) procedure is conducted to estimate the log probability of data Pr(X | K) (equation 12 in Pritchard *et al.* (2000)) for each value of *K*. STRUCTURE also calculates a proportional membership *Q* to each cluster (*K*) for each individual. STRUCTURE was applied without any prior information about the geographic origin of individuals using the following parameter settings: admixture model, correlated allele frequencies among populations, a burn-in period of 50'000 steps, and a chain length of $5x10^5$. The calculation for each *K* between 1 and 9 was performed 20 times.

Results

Demographic data

Of 813 banded nestlings, 44 (5.41 %) recruited within the study area. Of these, 55 % dispersed to a subpopulation other than their natal one. All subpopulations except the two smallest ones (Brüschweid and Feldbach) received recruits from a different subpopulation, and every subpopulation except Aathal contributed at least one recruit to another subpopulation. Of the 105 adults that bred in two subsequent years seven (6.67 %) bred in a different subpopulation in the second year. Across years, immigration rates were (1) maximally 61.37 % ± 0.06 (mean ± SD), (2) minimally 43.78 % ± 0.1, and (3) corrected for unbanded philopatric individuals 53.70 % ± 0.08. We did not observe any extinction of subpopulations.

Genetic data

All loci were highly polymorphic within each of the nine subpopulations (Table 3). Overall, a total of 144 alleles was found at 9 microsatellite loci, with an average of 16 ± 3.3 (mean ± SD) alleles per locus ranging from 11 alleles at Emb 17 to 22 alleles at Emb 112. Per subpopulation, the number of alleles averaged over loci was 10.3 ± 2.7 and varied from 6.2 in Brüschweid to 13.4 in Lützelsee. Mean allelic richness and observed heterozygosity ranged from 5.64 in Brüschweid to 6.05 in Feldbach und Uerzikon (mean ± SD, 5.91 ± 0.13) and from 0.796 in Brüschweid to 0.862 in Greifensee (0.842 ± 0.025), respectively. All subpopulations were in Hardy-Weinberg equilibrium (HWE) after Bonferroni correction except subpopulation Lützelsee (Table 3). We found no significant departure from HWE at total population level (locus combination Fisher's method: $\chi^2 = 24.9$, df = 18, $p > 0.124$). The magnitude of linkage disequilibrium measured as r_d ranged from -0.0041 in Brüschweid to 0.0329 in Aathal (mean ± SD, 0.0125 ± 0.013, Table 3), but only values for Bubikon and

Lützelsee were significant after the randomization procedure ($p < 0.009$). Linkage disequilibrium was not explained by subpopulation size ($R^2 = 0.01$, $F = 0.07$, $p = 0.798$).

The exact probability test showed a significant difference in allele frequencies among subpopulations (test combination Fisher's method: $\chi^2 = 72.1$, $df = 22$, $p < 0.001$). However, considering the overall F_{ST}-value of 0.005 (CI 95% 0.003 – 0.008) across subpopulations the degree of differentiation was very low. Calculation of pairwise F_{ST}-values generated almost only positive values, but all of them were smaller than 0.028 and not significant, except for the F_{ST}-value between Pfäffikersee and Uerzikon, which was significant after the permutation process followed by a Bonferroni correction (Table 4). No relation between geographic and genetic distances was found (Mantel test, $p = 0.209$). The cluster analysis performed with STRUCTURE indicated that most likely all subpopulations form one genetic cluster (that is $K = 1$, see Fig. 2). When regarding the proportional membership Q for the different values of K, each individual was assigned to each genetic cluster to nearly the same extent ($Q \approx 1/K$), also suggesting that all individuals belong to only one single cluster. This assignment was independent of an individuals' subpopulation of origin.

Discussion

Demographic data and the analyses of genetic variation within and among subpopulations of the reed bunting suggest that the spatial population structure of this species is consistent with the patchy population model. This model predicts regular exchange of individuals between all subpopulations, which we primarily found for juveniles. Accordingly, as predicted from the model, immigration into the study area was also high. The estimated immigration rate for the reed bunting fits within the range of values reported for other migratory (e.g. Great reed warbler, *Acrocephalus arundinaceus*: 54.6%, Hansson *et al.* 2004) and sedentary bird species (e.g. Willow tit, *Parus montanus*: 69.4%, Kvist *et al.* 2001; Orell 1999), for which high levels of gene flow among populations had also been shown (Bensch & Hasselquist 1999; Kvist *et al.* 2001). Unfortunately, we cannot distinguish whether immigrants came from the less-intensively monitored areas along Greifensee, Lützelsee, and Pfäffikersee or from populations farther away. However, in the former case, immigration from less-intensively to intensively monitored parts of the study area (see Methods) should have roughly equalled emigration from intensively to less-intensively monitored areas, but we did not find evidence for that. Only nine individuals moved from intensively to less-intensively monitored areas, indicating that almost all immigrants originated from outside the study area. The absence of extinctions or recolonizations further agrees with predictions from the patchy

population model. However, even if extinction risk of local subpopulations was high, five years of study may not have been sufficient to observe any extinctions or recolonizations, despite an average life expectancy of only two years (based on non-migratory British adults, Glutz von Blotzheim & Bauer 1997) and a generation time of 1.8 year (calculated following Lande *et al.* 2003). On the other hand, extinctions of subpopulations may be prevented by the high movement rates observed.

In line with the demographic data, our genetic data also support the patchy population model. According to this model, genetic diversity was high in all subpopulations, which suggests that all subpopulations were exposed to gene flow. The majority of the subpopulations was in Hardy-Weinberg equilibrium, as was the total population. We did not find any significant relationship between genetic and geographic distance, which also suggest that gene flow was high among subpopulations. However, lack of isolation-by-distance may also be explained by, for example, recent colonization processes, or a rapidly expanding population (Hutchison & Templeton 1999; Slatkin 1993). No correlation between the magnitude of linkage disequilibrium and subpopulation size was found, indicating that genetic drift is not a relevant factor at the level of subpopulations. Besides drift gene flow also could produce nonrandom associations of alleles resulting in a substantial magnitude of linkage disequilibrium, if immigrants were genetically different to local birds (Hedrick 2005). However, due to the small sizes of most subpopulations and the unknown immigration status of unbanded breeding individuals (see Methods), we were not able to calculate reliable estimates of subpopulation-specific immigration rates to test the influence of gene flow on the strength of linkage disequilibrium within subpopulations. When assigning individuals to one or more genetic clusters without a priori information on an individual's subpopulation of origin, we got clear evidence for only one genetic cluster in our study area.

One prediction of the patchy population model was only partly supported. In contrast to the prediction, we detected a significant difference in the allelic composition among subpopulations overall. In line with the prediction, on the other hand, pairwise comparisons indicated no significant differentiation between subpopulations in all but one case. However, both overall and pairwise Fst-values were always small, indicating very low magnitude of genetic differentiation. Slight differences among subpopulations could have been caused by small sample sizes, or by only a few immigrating individuals, if their population of origin had been genetically differentiated from the study population.

The genetic consequences of the three population models proposed would only be observed if the subpopulations have behaved as predicted by the models for some period of

time. For example, if connectivity among subpopulations had decreased very recently, no genetic differentiation between subpopulations would be observed, since our genetic estimates resemble past rather than current gene flow. However, our direct estimates of contemporary dispersal among subpopulations are in agreement with our genetic data on past gene flow, and combined they provide strong support for the patchy population model.

Both demographic and genetic results clearly rule out the isolated population model. This is in line with the general finding of weak genetic isolation between local bird populations (Walters 1998), and that occurrence of isolated populations may be restricted to species living on remote islands or to situations, in which distances between populations greatly exceed dispersal abilities (Pettersson 1985). Our data do not support the metapopulation hypothesis either. On the one hand, a patchy distribution of a species' habitat has often led to the a priori assumption that the species exhibits some form of metapopulation structure, not only when dispersal ability is restricted like, for example, in amphibians (Smith & Green 2005), but also in highly mobile species like migrating birds (Esler 2000; Hansson *et al.* 2002; Opdam 1991). Most studies rely on patch occupancy patterns to infer spatial population structure and end up suggesting evidence for metapopulation structure while genetic data are missing. However, studies having tested predictions for the distribution of neutral genetic diversity derived from metapopulation theory are rare. Evidence for metapopulation structure derived from genetic data has been found in plants (Tero *et al.* 2003), insects (Brookes *et al.* 1997), fishes (Garant *et al.* 2000), amphibians (Rowe *et al.* 2000) and mammals (Stewart *et al.* 1999). In birds, however, metapopulation structure derived from genetic data has only been shown for the capercaillie (*Tetrao urogallus*) in the Alps (Segelbacher & Storch 2002) and the Florida scrub-jay (*Aphelocoma coerulescens*, Coulon *et al.* 2008). There, genetic differentiation among subpopulations had been attributed to declines in subpopulation sizes caused by habitat deterioration owing to human land use. On the other hand and in contrast to metapopulation theory, Barrowclough (1983) has assumed that the high dispersal capacity of highly mobile taxa like birds will lead to admixture of populations despite the fragmentation of their habitat. Moreover, a spatially aggregated distribution of individuals does not necessarily demonstrate metapopulation structure, because restricted dispersal is not the only cause for localized groups of individuals of a taxon (Sutcliffe *et al.* 1997). Aggregated distributions can also occur in systems where individuals are highly mobile, but show some sort of aggregative behaviour in favoured patches (Koopman *et al.* 2007; Sutcliffe *et al.* 1997). This seems to be the case in our study system. The patchy distribution of breeding habitat in combination with the reed buntings'

narrow habitat preferences has not led to restricted dispersal or gene flow among subpopulations, low immigration or genetic structuring of subpopulations. Similarly, in many other migratory bird species lack of genetic structure has been found (Ball & Avise 1992; Kimura *et al.* 2002; Lovette *et al.* 2004; Mila *et al.* 2000; Ruegg & Smith 2002), even in species sensitive to habitat fragmentation (Veit *et al.* 2005).

The apparent lack of genetic structure in our study population may be explained by several hypotheses. First, birds are moving in response to habitat loss and/or fragmentation. It has been debated whether or not species change their dispersal behaviour following habitat loss and/or fragmentation (Ferraz *et al.* 2007; Opdam 1991). However, many examples exist were bird species did not disperse over increasing distances in response to habitat fragmentation (Cooper & Walters 2002; Matthysen 1999; Stouffer & Bierregaard 1995), whereas others did (Pasinelli *et al.* 2004; Van Houtan *et al.* 2007). Median natal dispersal distance in our study area was 4 km (interquartile range: 0.98 – 5.22 km, n = 30) and therefore more than four times larger than average dispersal distances reported for the reed bunting so far (Paradis *et al.* 1998). Increasing dispersal distances in response to habitat loss and/or fragmentation counteract population differentiation, which seems plausible for our study species when taking into account that the reed bunting is adapted to a naturally fragmented habitat, although levels of wetland fragmentation nowadays are undoubtedly far from natural. Second, migratory bird species have been shown to disperse longer distances than resident ones (Paradis *et al.* 1998), potentially leading to admixture of populations. The study reporting short dispersal distances for reed buntings (Paradis *et al.* 1998) has been conducted in the UK, where reed buntings do not migrate (Prys-Jones 1984), while reed buntings in Switzerland do. However, it has not only been shown that migratory bird species disperse further than resident ones (Paradis *et al.* 1998), but also that migrating populations disperse further and show weaker genetic differentiation than sedentary populations of the same species (Arguedas & Parker 2000). The greater dispersal distance of migratory populations has been attributed to pre-migratory dispersal behaviour, which may familiarize the birds with several sites, from which they might be able to choose, when returning from their wintering grounds the next breeding season. Thus, migratory behaviour may explain why reed buntings in our study area disperse irrespective of distance between subpopulations (no IBD), ultimately resulting in genetic admixture of subpopulations. Finally, the whole study population may be a sink sustained by one or more neighbouring populations acting as sources. If immigration rate is high, the study population would closely resemble the genetic constitution of its source population(s) resulting in genetic admixture (Gaggiotti & Smouse

1996). A source-sink situation on a regional scale (i.e. beyond the scale of our study) could therefore explain high immigration into and the apparent lack of genetic structure in our study population. In fact, demographic data indicate that the entire patchy population in our study area is a sink (Pasinelli *et al.* 2008b, submitted). Lack of genetic differentiation among subpopulations therefore indicates either unidirectional immigration from one source population, which could be genetically discrete, or immigration from several source-populations, which are connected by gene flow. However, if our study population is a sink population, it could weaken potential source populations by drawing individuals away from better sites (Gaona *et al.* 1998; Howe *et al.* 1991). On the other hand, wetland fragments in our study area may serve as a site of temporary residence for individuals that await an opportunity to disperse back to a source, in which case the presence of the sink may be beneficial for the species. Evaluation of these hypotheses will require more data on long distance dispersal and analysis of dispersal rates among our study population in the southeastern part of the Canton Zurich and neighbouring reed bunting populations at the border to Germany and in the middle and western part of the Swiss lowlands at least 50 km apart (Mayer *et al*. in prep.). Further studies are needed to confirm the general validity of our findings in terms of the patchy population model for other reed bunting populations and for other species with similar patterns of distribution and dispersal abilities.

Conservation implications

Knowing the spatial structure of local populations is not only of theoretical interest, but is also a crucial step in conservation planning for species living in a fragmented habitat (Lande & Barrowclough 1987). Our results give strong evidence for a genetically uniform patchy population consisting of several small breeding localities distributed according to available habitat. Despite high levels of habitat fragmentation, substantial contemporary dispersal and gene flow ensures connectivity among disjunct breeding localities. Consequently, there is only little risk for both local subpopulations and therefore also for the entire patchy population to go extinct. Conservation management should focus on the maintenance of this network of suitable breeding localities to promote the persistence of the patchy population. However, in the recent years, management of wetland reserves, particularly in the Canton Zurich, has focused on promoting species living outside reed beds such as orchids (*Orchidaceae*) or dragonflies (*Odonata*), preventing natural succession and eradicating exotic plant species (e.g. *Solidago* sp.). The intensive management leaves only narrow bands of old reed along water bodies and in some of the small wetland fragments, no

reed was left in some years. Consequently, those wetland fragments were not occupied by reed buntings in those years. Due to their high dispersal ability, reed buntings are capable of recolonizing wetland fragments immediately when habitat conditions have again become favourable. Ironically, in Canton Zurich management of wetlands for conservation seems to be the most important threat for the reed bunting. Since reproduction and demographic contributions of small and large wetland fragments did not differ (Pasinelli *et al.* 2008a) small fragments are of importance for the whole patchy population. Conservation management in favour of the reed bunting should therefore focus on the protection of all local breeding localities independently of size, to promote the persistence of the patchy reed bunting population in Canton Zurich and in other similarly fragmented landscapes.

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Tables

Table 1 Predictions about direct and indirect estimates of dispersal, the distribution of genetic variation and gene flow among local populations proposed from the three models of population structure '-': not observed/absent, '+': moderate level, '++': high level. Note that the patchy population model and the isolated population model represent extremes of a continuum of possible spatial population structure models, with the metapopulation model laying in-between the other two models.

	patchy population	metapopulation	isolated populations
Direct estimates			
Dispersal	++	+	–
Immigration	++	+	–
Extinctions / Recolonizations	– / –	+ / +	+ / –
Indirect estimates			
Genetic differentiation	–	+	+
Isolation by distance	–	+	–
Hardy-Weinberg equilibrium at total population level	+	–	–
Linkage disequilibrium	–	+*	+*
Gene flow	++	+	–
Number of genetic clusters	1	>1	>1[$]

* negatively related to subpopulation size, [$] equivalent to the number of subpopulations

Table 2 Location, size, number of fragments per subpopulations, coordinates, mean number of breeding pairs (BP) monitored per year, and samples collected from the nine subpopulations between 2002 – 2005.

Subpopulation	Size [ha]	Number of fragments	Coordinates	Mean BP / year	Sample size
Aathal	8.6	2	47°18'/08°45'	2.8 ± 1	10
Brüschweid	57.1	3	47°18'/08°48'	1.3 ± 0.5	6
Bubikon	46.4	4	47°16'/08°49'	7.5 ± 1.7	35
Feldbach	2.7	1	47°14'/08°48'	2.0 ± 0.8	7
Greifensee	44.1	1	47°19'/08°42'	12.0 ± 1.8	61
Lützelsee	58.9	3	47°16'/08°47'	12.3 ± 1.5	56
Nänikon	21.7	2	47°22'/08°42'	3.5 ± 0.6	13
Pfäffikersee	247.2	1	47°21'/08°47'	10.3 ± 1	44
Uerzikon	16.1	2	47°16'/08°46'	5.3 ± 1.7	21

Table 3 Estimates of average genetic diversity in the nine subpopulations. n = number of adults sampled, A = mean number of alleles per locus, R = mean allelic richness, observed (H_o) and expected (H_e) heterozygosity, HW = departure from Hardy-Weinberg expectations (n.s. = not significant, * = $p < 0.05$), mean F_{IS} per subpopulation and r_d (significant values are in bold).

Subpopulation	n	A	R	H_o	H_e	HW	F_{IS}	r_d
Aathal	10	8.56	5.95	0.849	0.842	n.s.	-0.009	0.0329
Brüschweid	6	6.22	5.64	0.796	0.825	n.s.	0.038	-0.0041
Bubikon	35	11.98	5.98	0.823	0.858	n.s.	0.042	**0.0313**
Feldbach	7	7.00	6.05	0.855	0.865	n.s.	0.013	0.0048
Greifensee	61	13.22	5.85	0.862	0.853	n.s.	-0.011	0.0065
Lützelsee	56	13.44	5.87	0.821	0.851	*	0.036	**0.0179**
Nänikon	13	9.22	5.96	0.880	0.861	n.s.	-0.022	0.0154
Pfäffikersee	44	12.65	5.82	0.837	0.850	n.s.	0.016	0.0065
Uerzikon	21	10.89	6.05	0.856	0.865	n.s.	0.011	0.0009

Table 4 Genotypic differentiation between pairs of subpopulations based on pairwise F_{ST} – values (below diagonal) and p-values from a G-based likelihood ratio test (above diagonal; after Bonferroni-correction p-values < 0.0014 are considered to be significant) for the nine reed bunting subpopulations (significant values are presented in bold).

	AA	BR	BU	FE	GR	LU	NA	PF	UE
Aathal (AA)	-	0.09583	0.04444	0.75556	0.00833	0.15833	0.64167	0.13333	0.00694
Brüschweid (BR)	0.0234	-	0.66944	0.73611	0.29167	0.08194	0.00972	0.03333	0.51806
Bubikon (BU)	0.0114	0.0025	-	0.91806	0.25556	0.19861	0.08194	0.00556	0.18333
Feldbach (FE)	-0.0033	-0.0079	-0.0091	-	0.31944	0.72917	0.28333	0.23333	0.3625
Greifensee (GR)	0.0078	0.0098	0.0055	0.0010	-	0.13889	0.16528	0.04167	0.07222
Lützelsee (LU)	0.0087	0.0213	0.0056	-0.0062	0.0022	-	0.31806	0.00278	0.09167
Nänikon (NA)	0.0090	0.0277	0.0057	0.0040	0.0035	0.0040	-	0.25278	0.08333
Pfäffikersee (PF)	0.0111	0.0152	0.0057	0.0054	0.0033	0.0075	0.0009	-	**0.00139**
Uerzikon (UE)	0.0145	-0.0033	0.0028	-0.0036	0.0052	0.0065	0.0100	**0.0126**	-

Figure legends

Figure 1

Location of the nine subpopulations with number of adults sampled per subpopulation. For details see Table 2.

Figure 2

Mean (± SD) likelihood values (Pr(X | K)) for each hypothesized genetic cluster (K).

Figures

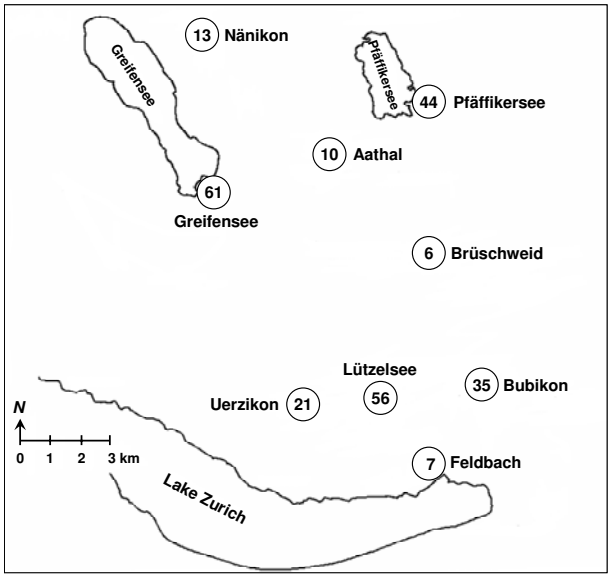

Fig. 1

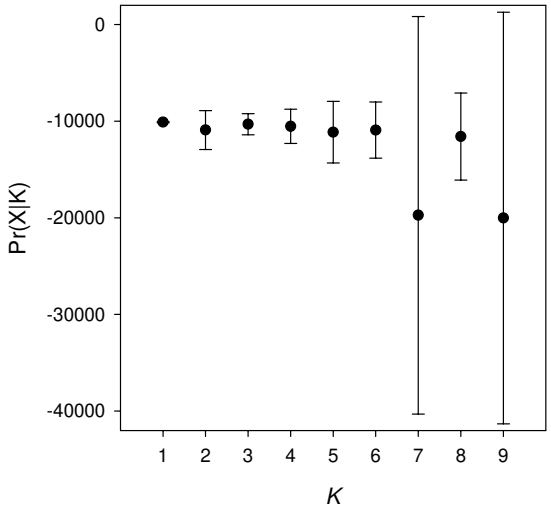

Fig. 2

CHAPTER 3

Regional abundance explains genetic diversity in small and large reed bunting populations

Christian Mayer, Karin Schiegg, Gilberto Pasinelli

Abstract

Populations are the traditional units for conservation and maintenance of genetic diversity, especially in small populations, has become a major concern in conservation biology. Reduction of genetic diversity due to genetic drift depends on population effective size, which is often correlated with local population size, i.e. population census or breeding size. However, if gene flow is high, the genetic boundaries of a local population may greatly exceed the geographic area originally used for population delineation. As a consequence, the predicted positive relationship between local population size and genetic diversity may not be found. In this study, we assess the importance of local population size and gene flow in relation to levels of genetic diversity in the reed bunting (*Emberiza schoeniclus schoeniclus*) across Europe. Distribution and local population sizes of this short-distance migratory songbird are restricted according to the distribution and size of wetlands. We show that genetic diversity of local reed bunting populations depended on regional abundance of the species rather than on local population sizes, which is in accordance with the high level of gene flow we found among reed bunting populations across Europe. Genetic diversity increased with latitude and was highest in Norway, the presumed centre of the species' distribution, and lowest in Switzerland and Austria, the southern edge of its distribution range. At the species' southern range margin, temporal variability in local population sizes may have reduced population long-term effective sizes and thus genetic diversity. A further reduction of genetic diversity may be counterbalanced if conservation efforts focus on the protection of remaining wetlands, enhancing habitat quality and thus regional population size.

Introduction

Populations are the traditional units for conservation. Conservation management is often concerned with the persistence of small populations because small populations face a high risk of extinction. Factors that increase extinction risk of populations include environmental and/or demographic stochasticity (Lande 1993) as well as genetic effects such as mutation accumulation, inbreeding, and loss of genetic diversity (Frankham 1995; Lynch *et al.* 1995; Spielman *et al.* 2004). The long-term persistence of populations depends on the individuals´ ability to respond to environmental change (Lande 1988; Reed & Frankham 2003), and genetic diversity is thought to allow populations to evolve in response to changing environmental conditions (Lande 1988; Meffe & Carroll 1997; Reed & Frankham 2003; Templeton *et al.* 2001). The maintenance of genetic diversity, especially in small populations, has therefore become a major concern in conservation biology.

How fast genetic diversity is lost depends on the strength of genetic drift, which is a direct function of N_e, the effective population size (Gillespie 2004). N_e approximates the average number of individuals that contribute offspring to the next generation (Beebee & Rowe 2004), and a decrease in N_e leads to a reduction in genetic diversity. Population genetic theory therefore predicts that populations with small effective sizes should show lower genetic diversity than large ones because of increased genetic drift in small populations (Hartl & Clark 1997). Because of the difficulties to determine N_e, population census or breeding size is often used as a correlate of N_e. Using census or breeding population size, reduced neutral genetic diversity has in fact been observed in many small natural populations (for review see Frankham 1996).

However, population census or breeding size, in the following referred to as 'local population size', may not always predict the level of genetic diversity. The reason is that definitions of local populations typically rely on qualitative descriptions (Waples & Gaggiotti 2006), such as the clustering of individuals of a species in a specific geographical area (Lapedes 1978) or habitat patch (Hanski & Gilpin 1996). These definitions neglect the genetic boundaries of local populations when gene flow occurs (Waples & Gaggiotti 2006). In the case of gene flow, the genetic boundaries no longer correspond to the geographic area or to the habitat patches used for the definition of local populations. As a consequence, local population size will no longer correlate with N_e and the relationship between local population size and genetic diversity is irrelevant. For example, van Rossum *et al.* (1997) found no relationship between local population sizes and genetic diversity of *Silene nutans* populations, because the effects of genetic drift reducing genetic diversity had been counterbalanced by

gene flow. Likewise, in a metapopulation of the butterfly *Melitaea cinxia* Saccheri *et al.* (1998) found that a decrease of genetic diversity in small local populations was prevented by high gene flow rates. These examples show that in cases of gene flow among populations, local population size may be an inadequate surrogate for N_e. Thus, in most situations it may be inappropriate to *a priori* assume reduced genetic diversity for small local populations when the genetic boundaries of these populations are unknown.

In cases of gene flow, the concept of the genetic neighbourhood proposed by Wright (1946; 1969) may be used to assess the relationship between N_e and genetic diversity. The size of the genetic neighbourhood is a correlate of N_e and defined as the number of individuals that mate at random within a single generation's dispersal distance (Wright 1946; Wright 1969). The genetic neighbourhood is therefore independent of *a priori*, area-based definitions of local populations. The size of the genetic neighbourhood depends on two components: the dispersal distance, which determines the geographic size of the neighbourhood, and the number of individuals within the geographic region of the neigbourhood. The size of the genetic neighbourhood is a measure of the abundance of individuals within the geographic region determined by the dispersal distance. However, the geographic extent of the genetic neighbourhood cannot be assessed when dispersal distances are unknown. In the following, we use the term 'region' for the geographically unknown extent of a local populations' genetic neighbourhood. Assuming that dispersal distances of individuals from different local populations are similar, the genetic neighbourhood size, and likewise N_e, increases with abundance of individuals within the region. The more dispersal distances of individuals from different populations vary, the less is this 'regional abundance' correlated with N_e. However, regional abundance may always be better correlated to N_e than local population sizes, when local populations are connected by gene flow.

Gene flow among local populations depends on a number of factors, the most important one being the dispersal capacity of a species. It has been assumed that gene flow is particularly high in birds owing to their generally high dispersal abilities (Barrowclough 1983). The low genetic structuring of many avian populations relative to non-avian vertebrates supports this assumption (Crochet 2000). However, examples exist, where the maximum potential for dispersal is not realized (e.g. Steiner & Gaston 2005), because social structure and individual behaviour prevents gene flow across larger geographic scales. These factors could make small, geographically isolated populations, even of species with high dispersal abilities, prone to the effects of genetic drift.

We present genetic data on the reed bunting (*Emberiza schoeniclus*), a short-distance migratory bird species. This small, socially monogamous passerine has a continuous Palaearctic distribution (Blümel 1995) with 3 subspecies inhabiting different biogeographical regions (Glutz von Blotzheim & Bauer 1997). In Central Europe, *Emberiza schoeniclus schoeniclus* prevails and occurs from 45° to 71° northern latitude. Despite this extensive range the subspecies often exists in geographically distinct small local groups of individuals usually referred to as populations. Due to the reed bunting's specific habitat requirements, the spatial distribution and size of these populations depend on the distribution and size of wetlands (Glutz von Blotzheim & Bauer 1997). Between the 1970s and early 1990s, considerable declines of the reed bunting have been reported for several European countries, for example Germany, Belgium, Finland and England (Heath et al. 2000). Especially in the intensively used agricultural landscapes of south and west Central Europe, habitat destruction has led to extinction of local populations (Glutz von Blotzheim & Bauer 1997) and to reductions in local population sizes (Blümel 1995), possibly making small local populations vulnerable to erosion of genetic diversity. However, it is unknown whether and how these small local populations are connected to surrounding reed bunting populations. Natal dispersal distances reported from ring recovery data of the British, non-migratory subspecies are small (Paradis *et al.* 1998), whereas ringing and molecular data from the migratory subspecies in Central European indicate more extensive dispersal at a local geographic scale (Mayer *et al.* 2009).

We examine whether small local reed bunting populations exhibit reduced levels of neutral genetic diversity, and if and how they are connected to other local populations on the European scale. By comparing genetic diversity among ten European reed bunting populations differing in breeding population size we investigate the importance of local population size for the maintenance of genetic diversity in this species. We predict that if gene flow between reed bunting populations is low, which might be the case if reed buntings are in fact poor dispersers (Paradis et al. 1998), then genetic diversity in local populations should be positively related to local population size, but not to regional abuncance. Alternatively, if reed bunting populations are well connected by gene flow, which is likely because migrants generally have high dispersal abilities (Crochet 2000; Rockwell & Barrowclough 1987), we predict genetic diversity in local populations to be positively related to regional abundance of the reed bunting, but not to local population size. Further, we investigated whether dispersal in the reed bunting was sex-biased, as in most other socially monogamous birds (Greenwood 1980).

Material and Methods

Ten geographically distinct populations of the reed bunting subspecies *schoeniclus* were included in our study (Fig. 1). These populations differed in local population sizes (Table 1), which we obtained in the following way. Local population sizes of the Zürcher Oberland (CH) and Baldeggersee (CH) were obtained from own census data. We extracted the breeding population size for the population Neuenburgersee (CH) from Glutz von Blotzheim & Bauer (1997), for Mettnau (D) and Altenrhein (CH) from Bauer & Heine (2005), for Neusiedlersee (AU) from Dvorak *et al.* (1997) and for the populations Lauwersemeer (NL) and Biesbosch (NL) from Meijer & Weel (2007). Censuses of the breeding population size of the populations Stawe Grabovnica (PL) were done in 1999, and of the population Øvre Heimdalen (N) in 2002. Census data were kindly provided by G. Buchanan (pers. com.) and O. Kleven (pers. com.), respectively. Blood samples were taken by trapping reed buntings with mist-nets during the breeding season (April – August, 1999 - 2005). Blood was absorbed with heparinized microcapillaries. Samples were either stored in heparinized microcapillaries or blown into APS-buffer (Arctander 1988) and then deep frozen at -20° C.

Laboratory analysis

DNA was extracted from blood samples with the Qiagen "Biosprint 96 DNA Blood Kit". We used 11 autosomal microsatellite loci for population genetic analysis, and four additional sex-specific (z-linked) microsatellite loci for analyses of population differentiation and sex-specific dispersal. Polymerase chain reaction amplification were conducted as described in Mayer *et al.* (2007). Amplified fragments were visualized on an ABI PRISM 3730 Avant capillary sequencer. Allele sizes were determined in relation to an internal size standard (GeneScan-500LIZ) using GENEMAPPER version 3.7.

Data analysis

Genetic variation

Genetic variation within each population was estimated using allele frequency data, from which the number of alleles per locus (A), the allelic richness (R) averaged over loci (Petit et al. 1998) and F_{IS} were calculated with FSTAT 2.93 (Goudet 2001). Expected and observed heterozygosities (H_e and H_o (Nei 1987) for each population were calculated with ARLEQUIN 3.1 (Excoffier et al. 2005). Departure from Hardy-Weinberg expectations of panmixia and from linkage equilibrium between all loci pairs was tested with probability tests

using GENEPOP on the web, available at http://genepop.curtin.edu.au/ (Raymond & Rousset 1995). These tests were conducted using a Markov chain with 5'000 batches each iterated 1'000 times and a dememorization number of 10'000 (Guo & Thompson 1992; Raymond & Rousset 1995). To avoid Type I statistical errors in multiple comparisons, a sequential Bonferroni correction was applied (Rice 1989).

Relation between genetic diversity and population size

We estimated genetic diversity with allelic richness (R), which is a measure of the number of alleles independent of sample size (El Mousadik & Petit 1996), and with H_e. These two measures of genetic diversity describe allelic data somewhat differently: R is affected by the presence of both rare and common alleles, while H_e is influenced predominantly by more common alleles. Population size was measured in terms of both local population size (Table 1) and regional abundance. However, data on regional abundance are difficult to obtain because this requires an *a priori* decision of what a region should be, within which the local population is embedded. However, reed bunting abundance has been reported to be positively correlated with latitude in Europe (Glutz von Blotzheim & Bauer 1997). We investigated the relationship between reed bunting abundance and latitude in the following way: for 16 countries in Europe (Belgium, Denmark, Germany, Estonia, Finland, France, Lithuania, Luxembourg, Netherlands, Norway, Austria, Poland, Sweden, Switzerland, Slovakia, Czech Republik and Hungary), reed bunting abundance was estimated by correcting census data for each country (Bufield & van Bommel 2004) for the country's land area (Berié et al. 2007). reed bunting abundance for each country was then regressed on the country's mean latitude. The regression was highly significant ($F = 12.89$, $df = 1$, $p = 0.003$), with 46.2% of the overall variance in abundance explained by latitude. Thus, we confirmed the association between regional abundance of reed buntings and latitude and therefore used the latitude of each of the ten geographically distinct local populations, for which we had genetic data, as a correlate for the respective regional abundance. Breeding population size and latitude of the ten European reed bunting populations, respectively, were related to genetic diversity by linear regressions using SPSS 12.0.2.

Genetic differentiation

Allele frequency differences between subpopulations were tested with an exact probability test using GENEPOP. Differentiation among subpopulations was described with F_{ST} calculated over all populations and for all population pairs according to Weir &

Cockerham (1984). F_{ST} – values were tested for departure from 0 by permuting genotypes among samples (5'000 permutations) with FSTAT 2.9.3.2. To avoid Type I statistical errors in multiple comparisons, a sequential Bonferroni correction was applied (Rice 1989). To assess whether geographical distance between populations explained genetic differentiation, isolation by distance was examined using a Mantel test (90'000 permutations) in ARLEQUIN 3.1 (Excoffier et al. 2005). Pairwise genetic distance defined as $F_{ST}/(1-F_{ST})$ was regressed on the logarithm of geographic distance in km. This regression is considered linear in a two-dimensional model (Rousset 1997).

Distinctiveness of populations was examined by clustering individual genotypes with the model-based clustering method of Pritchard *et al.* (2000) implemented in STRUCTURE 2.1. This method uses a Bayesian approach to detect potentially existing genetic structure without imposing any preconceived ideas of population substructure. The method assigns individuals, based on Hardy-Weinberg expectations, to a user-defined number of genetic clusters (*K*). A Markov chain Monte Carlo (MCMC) procedure is conducted to estimate the log probability of the data given K (equation 12 in Pritchard *et al.* (2000)) for each value of *K*. STRUCTURE also calculates a proportional membership *Q* to each cluster (*K*) for each individual. STRUCTURE was applied using the following parameter settings: admixture model, correlated allele frequencies among populations, a burn-in period of 10'000 steps and a chain length of 500'000. The calculation for each $K = 1 – 9$ was performed 20 times.

Sex-biased dispersal

To test for sex-biased dispersal F-statistics were applied to calculate the genetic variance that resides among populations for the two sexes separately (Goudet et al. 2002). Because allele frequencies for the more dispersing sex should be more homogeneous than those for the less dispersing sex, F_{ST} is expected to be higher in the less dispersing sex (Goudet et al. 2002). F_{ST} values for each sex across all populations were calculated from autosomal and z-linked loci separately using FSTAT 2.9.3.2. The probability that dispersal was unbiased by sex was then tested by assessing the significance of differences in F_{ST}-values between sexes for autosomal and z-linked loci using a randomization approach (10'000 randomizations).

Contemporary dispersal

We applied a molecular assignment test to identify potential immigrants using the Bayesian approach developed by Rannala & Mountain (1997) implemented in IMMANC 5.0.

Measures of historic gene flow, such as F_{ST}, rely on population models that assume a balance between forces of immigration, genetic drift, and mutation (Slatkin 1985). In contrast, molecular assignment tests are based on individual genotypes and do not depend on genetic equilibrium assumptions (Rannala & Mountain 1997). Contemporary dispersal is assessed by assigning each individual to the site in which its genotype is most likely to occur. The program estimates the probability densities for the allele frequencies of each population sampled, which are then used to calculate multilocus genotype probabilities for each population. Using a posterior probability ratio test and Monte Carlo simulations, each individual is then tested against the probability that it is an immigrant based on the multilocus genotype distributions calculated for each population. We assessed statistical significance with 10'000 replications per test using a rejection level (α) of 0.01 to reduce the probabilities of Type I errors (Rannala & Mountain 1997).

Results

Genetic variation

Both autosomal and z-linked microsatellite loci were highly polymorphic. In the autosomal loci (Table 2), the mean number of alleles (A) averaged across loci ranged from 9.45 in Baldeggersee (CH) to 16.45 in the population Zürcher Oberland (CH) (overall mean ± SD, 12.3 ± 2.1) and allelic richness (R) from 8.79 in Neuenburgersee (CH) to 10.03 in the Norwegian population (9.38 ± 0.39). Mean expected heterozygosity (H_e) was high and ranged from 0.844 in Biesbosch (NL) to 0.870 in Øvre Heimdalen (N) (0.861 ± 0.012) (Table 2). In the z-linked loci the mean number of alleles averaged across loci ranged from 7.5 in Biesbosch (NL) to 13.25 in the population Zürcher Oberland (CH) (mean ± SD, 9.38 ± 1.85), allelic richness from 6.66 in Biesbosch (NL) to 8.75 in the Norwegian population (7.52 ± 0.55). Mean expected heterozygosity (H_e) in z-linked loci was also high, ranging from 0.653 in Biesbosch (NL) to 0.785 in Øvre Heimdalen (N) (0.702 ± 0.04) (Table 2). All reed bunting populations were in Hardy-Weinberg equilibrium at autosomal loci (Table 2). Of 550 pairwise locus comparisons, linkage disequilibrium was detected for 24 comparisons. However, following a Bonferroni correction these tests were no longer significant (all $p > 0.05$).

Relation between genetic diversity and population size

Autosomal allelic richness increased with latitude ($R^2 = 0.69$, $F = 17.83$, $p = 0.003$; Fig. 2a) but not with local population size ($R^2 = 0.04$, $F = 0.31$, $p = 0.596$, Fig. 2c). When included in the same multiple regression model (Model: $R^2 = 0.70$, $F = 11.36$, $p = 0.006$), only

latitude ($t = 4.65$, $p = 0.02$), but not local population size ($t = 1.49$, $p = 0.181$), explained variation in allelic richness. Expected heterozygosity was neither significantly related to latitude ($R^2 = 0.17$, $F = 1.56$, $p = 0.242$, Fig. 2b) nor to local population size ($R^2 = 0.03$, $F = 0.23$, $p = 0.647$, Fig. 2d), and also in the multiple regression model H_e was neither related to latitude ($t = 1.24$, $p = 0.256$), nor to local population size ($t = 0.741$, $p = 0.483$).

Population differentiation

Overall, the distribution of allele frequencies differed significantly among populations (tests combination Fisher's method, autosomal loci: χ^2 = infinity, df = 22, $p < 0.001$; z-linked loci: χ^2 = infinity, df = 8, $p < 0.001$). For the autosomal loci, 12 out of 45 comparisons were significant (Table 3). Most significant pairwise comparisons included the population Zürcher Oberland (CH), which had the highest sample size, or population Øvre Heimdalen (N), which was most distant to all other populations.

The population Zürcher Oberland (CH) was differentiated from all populations except for its neighbouring populations Baldeggersee (CH), Mettnau (D) and Altenrhein (CH). The population Øvre Heimdalen (N) was clearly different from all other populations except for the populations Lauwersmee (NL), Baldeggersee (CH) and Altenrhein (CH). Significant genetic differences were also found between the population Neuenburgersee (CH) and the two populations Altenrhein (CH) and Neusiedlersee (AU) and between Neusiedlersee (AU) and Biesbosch (NL). When pairwise genetic differences between populations were estimated with the four z-linked loci, only six out of 45 comparisons were significant (Table 3), mostly reflecting the differentiation pattern revealed by the autosomal loci.

Despite significant differences between some of the populations the magnitude of differentiation was low when measured by F_{ST}. The overall F_{ST} value across autosomal loci was 0.009 (95% CI 0.007-0.011) and across z-linked loci 0.016. Pairwise F_{ST}-values ranged from -0.003 to 0.031 in the autosomal loci (Table 3) and from -0.017 to 0.058 in the z-linked loci (Table 3). A significant positive relation was detected between genetic distance and geographic distance in the autosomal loci (Mantel test, $r = 0.386$, $p = 0.047$, Fig. 3). In the z-linked loci, however, genetic distance was not significantly related to geographic distance ($r = 0.266$, $p = 0.124$).

In describing population genetic structure without *a priori* information about population boundaries, the cluster analysis performed in STRUCTURE put all individuals of the ten European reed bunting populations into one genetic cluster. The posterior probabilities $ln\ P(X|K)$ for each run of K showed the highest value for $K = 1$ (Fig. 4). Furthermore,

regardless of how many clusters *K* we assumed to be present in the dataset, the individual proportional membership *Q* to each cluster for a given *K* was ~1/*K,* meaning that most individuals were fairly admixed.

Sex-biased dispersal

For autosomal loci, F_{ST}-values did not differ significantly among males and females (n = 213 males / 195 females: F_{ST} = 0.01 / 0.0099, p = 0.452), while for the z-linked loci, males had slightly but not significantly higher F_{ST}-values than females (F_{ST} = 0.023 / 0.0161, p = 0.269). Thus, no sex-biased dispersal could be detected according to the comparison of F_{ST}-values for each sex obtained from autosomal or z-linked loci, respectively.

Contemporary dispersal

The power of the assignment tests was fairly high, ranging from 0.80 to 1.00 for all population pairs (mean ± SD, 0.98 ± 0.04) (Table 4). Thirty recent migrants were detected among 476 individuals from ten European populations. The population Zürcher Oberland (CH) received the highest number of immigrants (22). These immigrants consisted of migrant individuals from all other populations (Table 4).

Discussion

We showed that reed bunting populations across Europe are connected by gene flow despite loss and fragmentation of reed habitat. Due to high levels of gene flow, latitude as a proxy for regional abundance seems to be a good predictor of genetic diversity in the reed bunting, whereas local population size is not. According to a latitudinal increase in reed bunting abundance we found that genetic diversity was higher in northern than in southern reed bunting populations.

Relation between genetic diversity and population size

We did not find a relation between local population size and genetic diversity. Instead, latitude, which is correlated with regional abundance in the reed bunting, was positively related to genetic diversity. Together, these results imply that due to gene flow the genetic boundaries of reed bunting populations exceed the geographic area of local populations (which is corroborated by the high levels of gene flow among local populations, see below and Mayer *et al.* (2009)). Thus, regional abundance rather than local population size is a good correlate for effective population size in the reed bunting and therefore a good predictor for

genetic diversity. Our results are in line with the general theoretical expectation that predicts a positive relationship between effective population sizes and levels of genetic diversity (Frankham 1996; Wright 1931), as well as with empirical findings (Boessenkool *et al.* 2007; Johansson *et al.* 2006).

Of the two genetic diversity measures used, only allelic richness but not expected heterozygosity was positively related to latitude. However, the regression coefficient for the relation between expected heterozygosity and latitude was positive and not significantly different from the regression coefficient between allelic richness and latitude (Fisher's $Z = 1.334$, $p > 0.05$). This suggests that with a higher sample size than the current one a significant relation between latitude and expected heterozygosity might have been found as well. On the other hand, assuming the same mutation rate per individual in small and large populations, the average time between mutation and fixation of a new and therefore rare allele is proportional to the effective population size (Beebee & Rowe 2004). As a consequence, rare alleles are lost more quickly in small than in large populations. Accordingly, rare alleles are more frequent in regions with high reed bunting abundance and are lost more quickly in regions with low abundance. That we found a significant effect of latitude and therefore abundance on allelic richness may be due to the fact that this measure of genetic diversity is mainly influenced by the occurrence of rare alleles. In contrast, expected heterozygosity is mostly influenced by common alleles, whose probability to remain in the population is higher than that of rare alleles. That we did not find a correlation between expected heterozygosity and abundance therefore suggests that the impact of genetic drift was weak in the reed bunting.

Gene flow and contemporary dispersal

In contrast to ringing recovery data suggesting only low dispersal in the reed bunting (Paradis et al. 1998), our genetic data revealed little differentiation between reed bunting populations across Europe. Pairwise genetic differentiation was not significant except for comparisons involving the Zürcher Oberland (CH) and Øvre Heimdalen (N). The significance of F_{ST}-values involving the Zürcher Oberland (CH) may be most likely caused by the very high sample size for that population. However, pairwise comparisons involving the Norwegian population with comparatively low sample size, together with the pattern of isolation-by-distance suggest that geographic distance between populations explains genetic differentiation. Despite significant differentiation between geographically distant populations, pairwise F_{ST}-values were on average low, indicating relatively high levels of gene flow even

on a continental scale, as has been predicted for highly mobile species like migratory birds (Barrowclough 1980; Rockwell & Barrowclough 1987). The Bayesian cluster analysis also confirmed high rates of genetic exchange between populations, suggesting that all sampled individuals belonged to a single genetic population. However, in the situation of isolation-by-distance, allele frequencies vary gradually across regions (Beebee & Rowe 2004). The underlying model implemented in STRUCTURE is not well suited to data from this kind of scenario and interpretation of the results may be challenging (Pritchard & Wen 2003). To account for the effect of isolation-by-distance on the allele frequency distributions, we again performed a cluster analysis including only the three reed bunting populations, for which we got the clearest signal of genetic differentiation (Zürcher Oberland, Neuenburgersee (CH), and Øvre Heimdalen (N)). However, also in this analysis STRUCTURE suggested only one genetic cluster (data not shown). High levels of gene flow across large geographic scales, such as we report here, are in line with empirical findings for other European (Bensch *et al.* 1999; Perez-Tris *et al.* 2004) and neotropical migrant songbirds (Ball & Avise 1992; Davis *et al.* 2006; Zink & Dittmann 1993), which also show only little genetic differentiation even when breeding habitats have been lost and extensively fragmented (Veit *et al.* 2005).

Results of the assignment tests confirmed that contemporary dispersal also seems to occur. We therefore argue that in reed buntings habitat loss and fragmentation due to wetland destruction have not led to isolation even of small local populations, for which immigration is generally predicted to be less likely to occur than into larger populations (Caughley 1994). That most immigrants were detected in the Zürcher Oberland (CH) may be due to the relatively large number of sampled individuals compared to the other European populations and does therefore not preclude that contemporary dispersal between the other investigated populations is in reality higher than estimated in this study (Rannala & Mountain 1997).

Sex-biased dispersal

In contrast to Greenwood's (1980) prediction of female-biased dispersal in socially monogamous birds, which was confirmed by Clarke *et al.* (1997), we did not find any significant difference in F_{ST}-values between the sexes in the reed bunting. Instead, our results indicate that genetic exchange between populations is due to dispersal of both sexes. In the sex-specific z-linked microsatellite loci, alleles spend twice as much time in males (ZZ) as they do in females (ZW). If females disperse more than males, the signal of genetic differences in dispersal between sexes should be stronger in the z-linked than in the autosomal microsatellite loci. However, even in the z-linked microsatellite loci we could not detect any

significant differences in F_{ST}-values between the two sexes. In accordance with our results, capture/recapture data on reed bunting dispersal collected within the Zürcher Oberland (CH) did not reveal any differences in dispersal distance between sexes (G. Pasinelli, unpublished). Similarly, no sex-biased dispersal has been observed in some other socially monogamous bird species (see Clarke *et al.* (1997) for review).

Possible causes of a latitudinal cline in genetic diversity

Quaternary climatic fluctuations have been identified as the main historical processes that shaped the geographic distribution and genetic diversity of many taxa in the temperate Northern Hemisphere (Frenzel 1973; Hewitt 2004). Contemporary latitudinal patterns of genetic diversity may therefore largely be the result of a recolonization of central and northern Europe from Mediterranean refugia, because sequential founder events during recolonization have led to loss of genetic diversity at higher latitudes (Hewitt 2004). Decreasing genetic diversity towards the north has been reported for many taxa including, for example, plants (Lagercrantz & Ryman 1990) and birds (Merilä *et al.* 1996). However, the latitudinal increase in genetic diversity we found in the reed bunting is somewhat unexpected when compared to these historic colonization processes reported for temperate European species. For example, if the observed pattern of decreasing genetic diversity towards the south is a relict of a Pleistocene colonization process, we have to assume a northern refugium from which colonization has taken place. Northern refugia have in fact been identified, although mainly for cold-tolerant species (Stewart & Lister 2001). The reed bunting is not considered to be cold-tolerant (Blümel 1995; Glutz von Blotzheim & Bauer 1997). Furthermore, it is unlikely to assume a northerly situated refugium for a bird that migrates towards south-western Europe, if migratory directions were constrained by colonization routes, as populations would tend to winter in their original breeding grounds (Ruegg & Smith 2002; Safriel 1995). Thus, it is more likely that in the reed bunting the imprint of a postglacial colonization from southern refugia on the geographic distribution of genetic diversity has vanished due to processes resulting from the climatic changes affecting the reed bunting's habitat during the postglacial. After retreat of glaciers, wet conditions prevailed at southern latitudes, resulting in the formation of wetlands. As a consequence of the increasingly warmer climate with time after glaciation, these southerly wetlands deteriorated or were completely lost as habitats for the reed bunting. A following decline in reed bunting abundance due to suboptimal habitat conditions in the south could then have increased the impact of genetic drift reducing genetic diversity and shaping the geographic distribution of genetic variability into the latitudinal

pattern observed today. This explanation is consistent with biogeographical models such as the central-marginal hypothesis that predicts populations at the species' geographic range margin to be less genetically diverse than populations at the centre of a species' range (Eckert et al. 2008). The central-marginal hypothesis is based on the assumption that a species is most abundant at the centre of its geographic range, where survival, reproduction and population growth are highest and is expected to become less abundant where conditions depart from this optimum (Brown 1984). This implies that effective population sizes are highest at the range centre and lowest at the range margins. As a result, populations at the periphery of a species' geographic range exhibit reduced genetic diversity, which has been shown for many species (Eckert *et al.* 2008; Merilä *et al.* 1997; Vucetich & Waite 2003). However, it is unclear whether the pattern of genetic diversity observed today solely reflects postglacial habitat alteration and/or additional contemporary processes such as habitat destruction and fragmentation due to agricultural land use resulting in current population sizes.

Conservation implications

Local populations of the reed bunting are interconnected through high levels of gene flow, making regional abundance a much better predictor of genetic diversity than local populations size. Since gene flow among populations is high, even over large geographic scales, the persistence of local small populations is more likely to depend on demographic and environmental stochasticity (Lande 1988; Willi *et al.* 2006) than on genetic constraints. The strong dispersal ability of the reed bunting may be of advantage when small local populations have gone extinct, because unsettled habitats may easily be recolonized. Both genetic diversity and regional abundance increased with latitude, suggesting that the centre of the species' range is located at northern latitudes. Besides the reduced abundance at the species' southern range margin, temporal variability in population sizes, which has been expected (Caughley et al. 1988) and shown to be high in marginal populations (Curnutt et al. 1996), further reduces population long term effective sizes and thus genetic diversity. If conservation efforts, especially at the southern range margin, can achieve the maintenance of small local reed bunting populations by enhancing habitat quality and thus reproductive success and by protecting remaining wetland habitats as well, regional abundance could be increased, thereby counteracting further reduction of genetic diversity. In fact, even small local populations of the reed bunting may contribute to this process, since small wetland fragments have been shown to be demographically equal to large ones (Pasinelli *et al.* 2008; Pasinelli *et al.*

accepted) and can therefore be valuable for the maintenance of reed bunting population networks.

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Tables

Table 1 Locations of the ten European reed bunting populations, coordinates, mean number of breeding pairs (BP) per year, and number of blood samples.

Site	Coordinates	BP / year	Blood samples
Zürcher Oberland (CH)	47.299°/8.766°	120	253
Baldeggersee (CH)	47.211°/8.244°	60	14
Mettnau (D)	47.729°/9.003°	500	29
Altenrhein (CH)	47.494°/9.554°	170	21
Neuenburgersee (CH)	46.899°/6.914°	800	21
Lauwersmeer (NL)	53.333°/6.250°	4500	17
Biesbosch (NL)	51.751°/4.803°	2100	20
Neusiedlersee (AU)	47.724°/16.769°	5500	40
Stawe Grabovnica (PL)	52.473°/16.893°	80	34
Øvre Heimdalen (N)	61.191°/8.647°	100	27

Table 2 Genetic variation in ten European reed bunting populations based on 11 autosomal microsatellite loci (upper values per cell) and 4 z-linked loci (lower values). n = number of individuals, A = mean number of alleles per locus, R = mean allelic richness, expected (H_e) and observed (H_o) heterozygosities, HW = departure from Hardy-Weinberg expectations (n.s. = not significant) and mean F_{IS} per population.

Population	n	A	R	H_e	H_o	HW	F_{IS}
Zürcher Oberland	253	16.45	9.08	0.860	0.835	n.s.	0.029
(CH)		13.25	7.5	0.720	0.387		0.463
Baldeggersee	14	9.45	9.08	0.846	0.852	n.s.	-0.008
(CH)		7.75	7.4	0.732	0.493		0.335
Mettnau	29	12.18	9.31	0.868	0.851	n.s.	0.019
(D)		9.5	7.9	0.689	0.348		0.499
Altenrhein	21	11.18	9.33	0.865	0.801	n.s.	0.077
(CH)		8.25	7.2	0.730	0.407		0.45
Neuenburgersee	21	10.55	8.99	0.840	0.836	n.s.	0.009
(CH)		8.25	7.4	0.665	0.455		0.321
Lauwersmeer	17	11.36	9.96	0.882	0.854	n.s.	0.032
(NL)		7.75	7.1	0.694	0.341		0.516
Biesbosch	20	10.91	9.24	0.844	0.822	n.s.	0.027
(NL)		7.5	6.7	0.653	0.413		0.375
Neusiedlersee	40	13.73	9.48	0.866	0.848	n.s.	0.021
(AU)		10.5	7.5	0.666	0.343		0.488
Stawe Grabovnica	34	13.73	9.48	0.863	0.828	n.s.	0.042
(PL)[$]		10	7.8	0.689	0.669		0.03
Øvre Heimdalen	27	13.18	10.03	0.870	0.871	n.s.	-0.001
(N)		11	8.8	0.785	0.503		0.365

[$] only male data available

Table 3 Pairwise genotypic differentiation of ten European reed bunting populations. Numbers above the diagonal are p-values (bold: significant after Bonferroni correction), numbers below the diagonal are pairwise F_{ST}-values. In each cell, the upper values refer to results from 11 autosomal microsatellite loci, the lower values to the four z-linked loci.

Population	ZO	BA	ME	AL	NE	LA	BI	AU	PO	NO
Zürcher Oberland	-	0.082	0.002	0.010	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**
(ZO)		0.102	0.524	0.719	0.051	0.006	**0.001**	**0.001**	0.002	**0.001**
Baldeggersee	0.003	-	0.064	0.090	0.076	0.176	0.011	0.031	0.033	0.003
(BA)	0.007		0.846	0.947	0.068	0.811	0.097	0.230	0.016	0.002
Mettnau	0.002	0.010	-	0.392	0.004	0.121	0.004	0.113	0.333	**0.001**
(ME)	0.005	0.001		0.578	0.197	0.300	0.101	0.052	0.060	0.002
Altenrhein	0.006	0.011	0.003	-	**0.001**	0.241	0.042	0.191	0.351	0.007
(AL)	-0.003	-0.017	0.007		0.201	0.151	0.052	0.088	0.096	0.006
Neuenburgersee	0.013	0.008	0.009	0.020	-	0.003	0.027	**0.001**	0.006	**0.001**
(NE)	0.010	0.015	0.016	-0.002		0.247	0.029	0.091	0.121	0.011
Lauwersmeer	0.004	0.011	0.005	0.005	0.015	-	0.049	0.244	0.836	0.223
(LA)	0.011	-0.009	0.012	0.005	0.005		0.042	0.118	**0.001**	0.006
Biesbosch	0.011	0.007	0.007	0.008	0.006	0.011	-	**0.001**	0.161	**0.001**
(BI)	0.026	0.016	0.023	0.018	0.020	0.026		0.518	0.078	**0.001**
Neusiedlersee	0.008	0.009	0.003	0.007	0.014	0.001	0.011	-	0.569	**0.001**
(AU)	0.023	0.008	0.021	0.013	0.015	0.015	-0.004		0.044	**0.001**
Stawe Grabovnica	0.008	0.012	0.004	0.003	0.011	-0.003	0.004	0.000	-	**0.001**
(PO)	0.013	0.012	0.016	0.004	0.003	0.016	0.012	0.009		0.006
Øvre Heimdalen	0.020	0.018	0.021	0.015	0.031	0.005	0.017	0.016	0.013	-
(NO)	0.033	0.021	0.037	0.020	0.033	0.024	0.058	0.048	0.019	

Table 4 Probability of detecting immigrated individuals based on 476 reed bunting blood samples. Rows indicate the population in which an individual was sampled, while columns indicate the most likely source population of the sampled individual. The number of immigrants detected for each population pair is given in parentheses.

	Individual originated from									
Individual sampled from	ZO	BA	ME	AL	NE	LA	BI	AU	PO	NO
Zürcher Oberland (ZO)	-	0.95 (1)	0.80 (3)	0.97 (3)	0.95 (3)	0.95 (4)	0.98 (1)	0.87 (3)	0.90 (2)	0.99 (2)
Baldeggersee (BA)	0.96	-	0.99	1.00	1.00	1.00	1.00	1.00	0.99	1.00
Mettnau (ME)	0.84	0.99	-	0.99	0.99	0.98	1.00	0.94 (1)	0.92	1.00
Altenrhein (AL)	0.96	1.00	0.99	-	1.00	1.00	1.00 (1)	0.98 (1)	0.99	1.00
Neuenburgersee (NE)	0.96	1.00	0.99	1.00	-	1.00	0.99	0.99	0.99	1.00
Lauwersmeer (LA)	0.94	1.00	0.99	1.00	1.00	-	1.00	0.98	0.97	0.99
Biesbosch (BI)	0.98	1.00	0.99	1.00	0.99	1.00	-	0.99	0.98	1.00
Neusiedlersee (AU)	0.86	0.99	0.94	0.98	0.98 (1)	0.98	0.99	-	0.91	0.99
Stawe Grabovnica (PO)	0.90 (1)	1.00	0.91	0.99 (1)	0.99	0.96	0.98	0.90	-	0.99 (1)
Øvre Heimdalen (NO)	0.99	1.00	1.00	1.00	1.00	0.99 (1)	1.00	1.00	0.99	-

Figure legends

Figure 1

Locations of the ten European reed bunting populations.

Figure 2

Relationships between latitude and allelic richness (a) and expected heterozygosity (b) for ten European reed bunting populations.

Figure 3

Relation between genetic distance (calculated as $F_{ST}/(1-F_{ST})$) and geographical distance (ln kilometers) obtained from pairwise comparisons between ten reed bunting populations.

Figure 4

Mean and SE of the posterior probabilities ($ln\ P(X|K)$) of the cluster analysis performed for ten European reed bunting populations calculated by STRUCTURE for 20 independent runs of each K, assuming an admixture model and correlated allele frequencies.

Figures

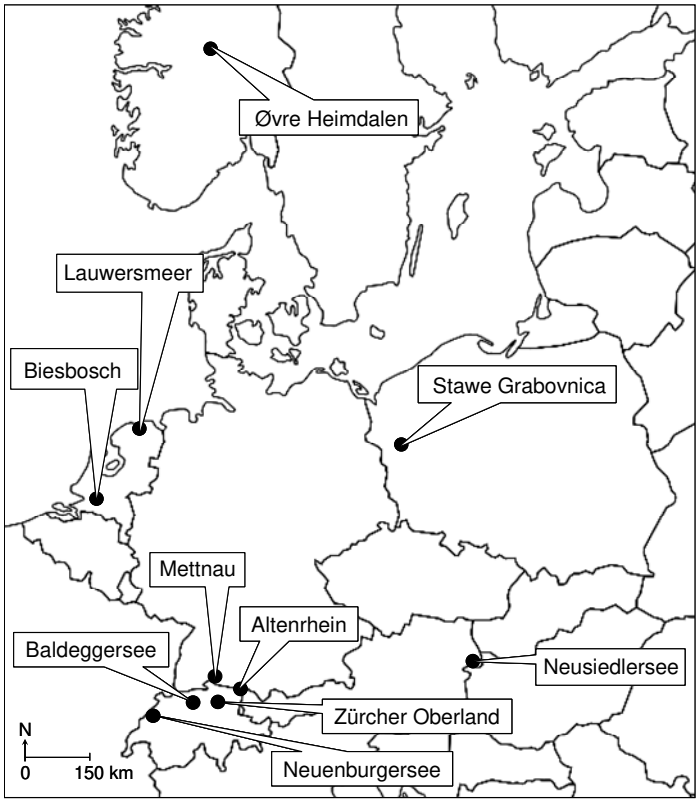
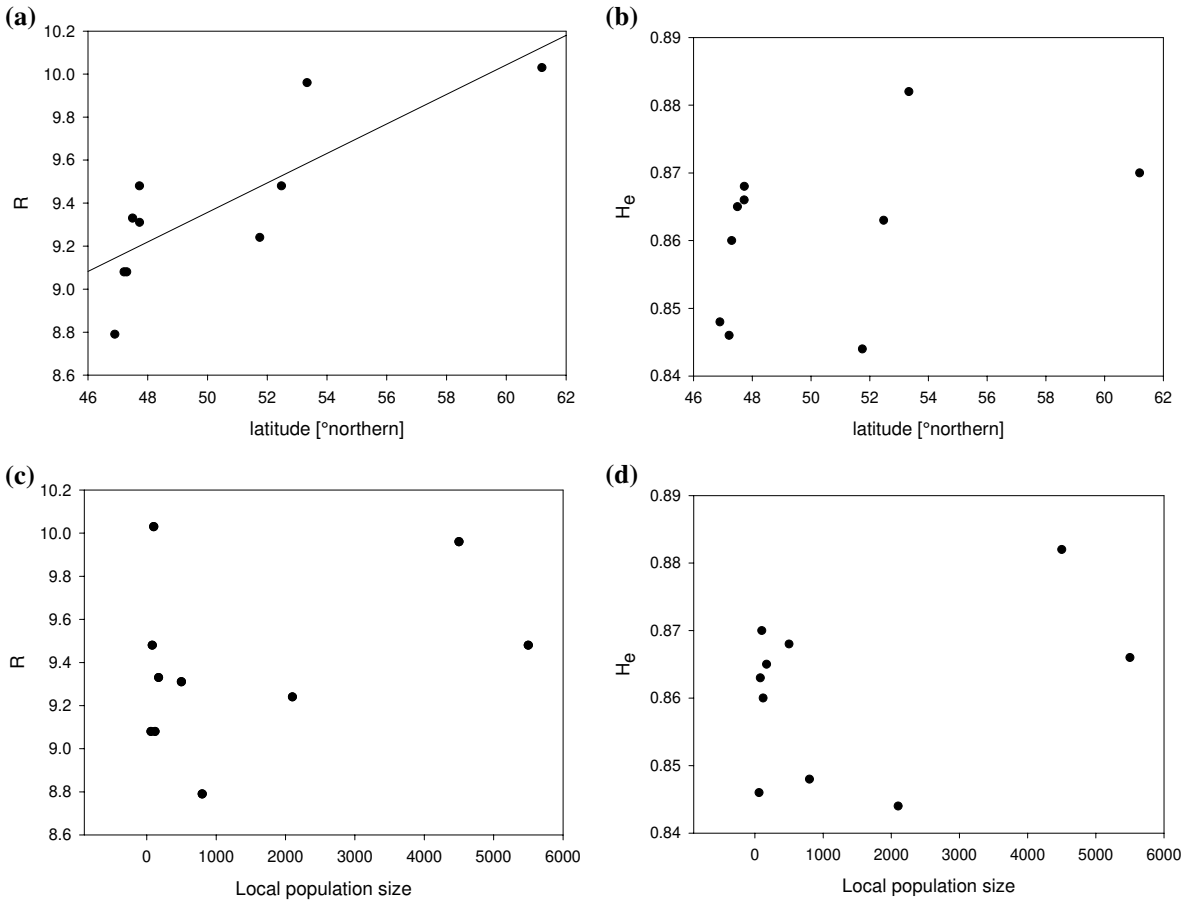


Fig. 1


(a)
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(b)
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latitude [°northern]
(c)
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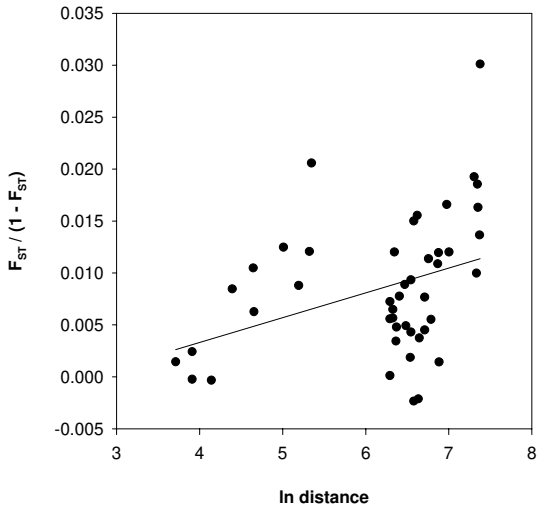
Local population size
(d)
He
Local population size

Fig. 2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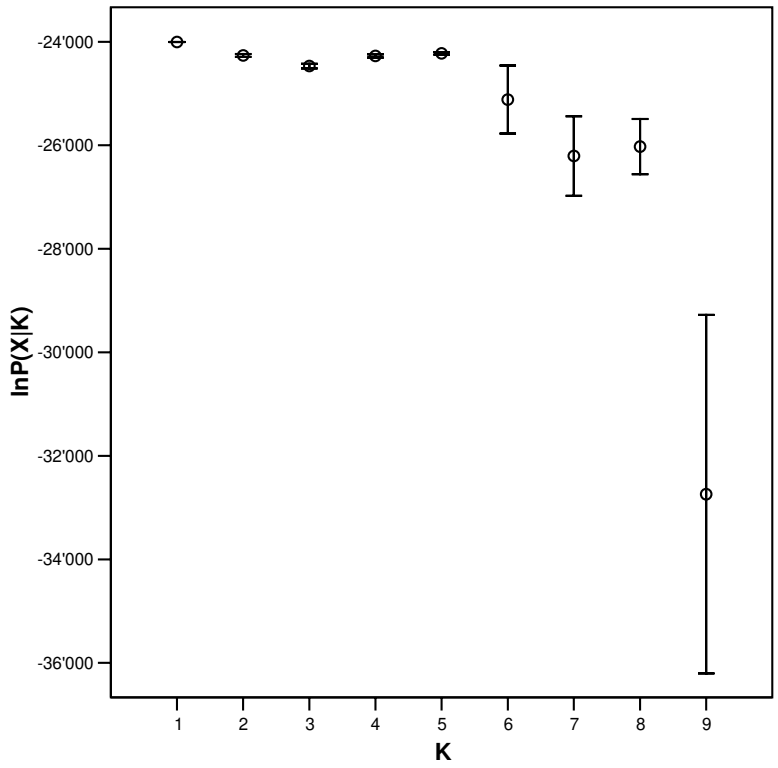
F_{ST} / (1 - F_{ST})
ln distance

Fig. 3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-28'000
-30'000
-32'000
-34'000
-36'000
lnP(X|K)
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2
3
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5
6
7
8
9
K

Fig. 4

CHAPTER 4

New support for an old hypothesis: density affects extra-pair paternity

Christian Mayer & Gilberto Pasinelli

Abstract

Density has been suggested to affect variation of extra-pair paternity (EPP) in avian mating systems, because it increases encounter rates and mating opportunities when individuals search for potential extra-pair mates. So far, the significance of density affecting EPP variation in intraspecific comparisons remained controversial. However, the absence of the predicted effect in within- and among-population comparisons as revealed by many empirical studies may mostly be attributed to potentially confounding factors and poor study design. Density measures may not always reflect extra-pair mating opportunities, mate guarding efforts may vary with density, different migration distances and climatic conditions could cause population differences in EPP, and low variation in density and small sample sizes weaken the test power. Taking all those factors into account, we tested if EPP rates within and among local populations of the reed bunting (*Emberiza schoeniclus*) were related to density. Our analyses were based on data from 18 local populations studied over four years. Within populations, the proportion of extra-pair young (EPY) in broods was positively related to local breeding density. Similarly, among local populations, proportion of EPY was positively associated with population density. We also show that EPP was absent where populations consisted of a single breeding pair, i.e. when no extra-pair mating opportunities were available. Our study confirms that density is an important biological factor, which significantly influences the amount of EPP within and among populations, but also supports the view that other mechanisms influence EPP variation on top of the variation explained by density.

Introduction

Variation in population density is one of the traditional factors proposed to explain inter- and intraspecific variation in extra-pair paternity (EPP) in avian mating systems. However, the density hypothesis has fallen into disfavour because there is little evidence for a general relationship between population density and EPP across species (Griffith *et al.* 2002; but see Moller & Ninni 1998; Westneat & Sherman 1997). Briefly, the density hypothesis states that increased proximity among individuals increases encounter rates and mating opportunities when searching for potential extra-pair mates. This should reduce the costs of extra-pair matings, so density if density increases the rate of extra-pair paternity should increase, too (Westneat et al. 1990). However, even on the intraspecific level the effect of density on EPP has turned out to be less important (Bennett & Owens 2002; Griffith *et al.* 2002; Westneat & Sherman 1997) than proponents of the hypothesis initially envisioned (Birkhead 1979; Westneat *et al.* 1990). We argue that it may be premature to reject the density hypothesis because most studies have not been designed to properly test it (Griffith et al. 2002), and a relationship between EPP and density may have often been masked by interacting factors (Westneat & Sherman 1997; Westneat & Stewart 2003). These factors have been discussed but not accounted for in previous studies that did not support the density hypothesis.

One problem with previous tests of the density hypothesis is that density estimates may not reflect the opportunity for extra-pair matings. If extra-pair copulation (EPC) behaviour mainly occurs in the area around a territory, then local breeding density is likely to affect extra-pair mating opportunities. In contrast, if EPCs take place well beyond the immediate territory neighborhood, and males and females encounter each other at common sites (e.g. Dunn *et al.* 1994b; Reyer *et al.* 1997), local breeding density or territory structure is unlikely to be related to variation in extra-pair paternity. The rate of EPCs might also be decoupled from local breeding density if non-territorial floater males are common (Ewen *et al.* 1999; Tarof *et al.* 1998) or if the species is not territorial (Dunn & Whittingham 2007; Griffith *et al.* 1999; Westneat & Stewart 2003).

A second problem discussed in previous studies is that mate-guarding efforts may be more effective and/or increase at high density. Males may be more likely to successfully prevent extra-pair encounters of their social females if more crowded habitats are visually less occluded. The potentially confounding effect of habitat structure on mate-guarding success may be strong only when comparing across populations (Westneat & Mays 2005). Another possibility is that social males invest more effort preventing extra-pair matings of their

females at increased densities (Komdeur 2001). In this case, mate guarding could compensate for a density-dependent increase in opportunity for EPP (Kokko & Rankin 2006).

A third interacting factor is the difference in migration distances among populations studied. The reasoning is that long migration distances increase the ecological need to settle quickly resulting in inaccurate or hasty mate choice. As a consequence, the proportion of high quality females paired to low quality males may increase, which enhances the benefits to females of pursuing EPCs (Weatherhead & Yezerinac 1998). Long migration distance may thus increase the level of EPP in populations at higher latitudes (Spottiswoode & Moller 2004) and could therefore obscure the effect of density on EPP when populations at different latitudes are compared.

Fourth, variation in local breeding density may have been insufficient in previous studies to find an effect on EPP. A relationship between density and EPP is not predicted if density exceeds a threshold level resulting in sufficient extra-pair partners at all local breeding densities (Westneat *et al.* 1990). Similarly, a relationship between density and EPP should not occur when densities are so low that potential extra-pair mates do not encounter one another (Orell et al. 1997).

A final problem is that previous studies typically had insufficient sample sizes. The reliability of estimated EPP rates is greatly influenced by sample size. In their review, Griffith et al. (2002) calculated that 99% confidence intervals of EPP estimates extended up to 30% beyond the estimate itself when less than 100 offspring were sampled, which was the case in more than half of the studies reviewed. Comparisons between populations are therefore afflicted with a high level of uncertainty, especially when considering that 75% of published EPP estimates fall between zero and 20% (Fig.1 in Griffith et al. 2002).

Most previous tests of the density hypothesis are vulnerable to at least one of these problems. The majority of studies compared differences in EPP rates between individuals within the same population, and all studies on EPP in relation to density across populations involved a small number of populations only (Griffith *et al.* 2002). To assess the relation between population density and variation in EPP rates within and among populations, it is essential to include a reasonable number of populations and to study systems where the predicted effect is not obscured by interacting factors (Westneat & Sherman 1997).

Here, we present data on density and EPP rates from multiple local populations of the reed bunting (*Emberiza schoeniclus*) in Switzerland. We test two predictions of the density hypothesis. First, we predict that levels of EPP within populations are positively related to local breeding density, assessed through measures at the territory level. Second, we expect

that levels of EPP among populations are positively related to breeding density, assessed at the level of the local population. Our system is well-suited for testing the density hypothesis because first, the species appears to avoid the pitfalls mentioned above (see next section), and second, our study setup allows to compare among many populations with varying densities within a local geographic area.

The reed bunting is a small socially monogamous short distance migrant restricted to wetlands (Glutz von Blotzheim & Bauer 1997). High levels of extra-pair paternity (up to 55% extra-pair young in 86% of broods) have been reported from populations throughout Europe (Bouwman *et al.* 2005; Dixon *et al.* 1994; Kleven & Lifjeld 2005; Suter *et al.* 2007). The reed bunting defends only nesting territories (Glutz von Blotzheim & Bauer 1997). Both sexes forage outside these territories. In spite of this, EPP has been shown to occur among close neighbours in most cases (Bouwman & Komdeur 2006) and floaters apparently are rare (own observation). Consequently, density estimates at the level of the territory are likely to reflect and hence correlate with extra-pair mating opportunities. Adults forage in open habitat (Marthinsen et al. 2005) and nest cryptically within old, rather dense reed beds (*Phragmites* sp.), where vision is frequently obstructed (Pasinelli & Schiegg 2006). Reed bunting mate-guarding efforts do not vary with density (Marthinsen et al. 2005). In our study, then, neither habitat structure nor mate-guarding efforts are likely to vary with density potentially masking a density-dependent response in EPP rate. We studied 19 local populations scattered within a small area in the Swiss lowlands, so any potential effect of migration distance on EPP variation among populations is negligible. Numbers of breeding pairs in the local populations studied ranged from 1 - 50, and accordingly, variation in breeding density among populations was high. Four years of sampling (2002-2005) resulted in data on extra-pair paternity of 669 nestlings from 181 broods. This sample size is large enough to provide reliable estimates of extra-pair paternity rates (Griffith et al. 2002).

Material and Methods

Field work

The study was carried out in 19 wetland nature reserves scattered over an area of 200 km^2 in south-eastern Canton Zurich, Switzerland. The reserves range in size from 1.9 to 247.2 ha (median 10.5 ha, interquartile range 4.2-16.7 ha) and represent all potentially suitable breeding localities for reed buntings within this region (Table 1). With respect to extra-pair mating behaviour, each wetland reserve was considered as one local population. From 2002 to 2005 we monitored all breeding pairs in all populations, except for the three largest

(Greifensee, Lützelsee and Pfäffikersee), where we surveyed at least 10 breeding pairs annually in the same study plots randomly chosen in 2002. Monitoring took place from early March, when males return from their wintering grounds, to early August, when the breeding period ends. Nests were located by observing females building their nest, leaving their nest and returning to it during incubation, or when feeding their young. The young were banded between nestling day 6 and 9, with each nestling obtaining a numbered aluminium ring and a unique combination of three coloured plastic rings allowing individual identification in the field. After fledging or nest loss, nest locations were recorded using a hand-held global position system (GPS) receiver (GPS-12XL with RXMAR decoder, Garmin, Olathe, KS; GoeExplorer 3, Trimble, Sunnyvale CA; Leica GS50, Leica, St. Gallen, Switzerland). The precision of the GPS locations after differential correction was ≤ 2 m. Adults were colour-marked in the same way as nestlings, and social parents were determined by observation of colour-ringed individuals during nest building, incubation and the nestling period. Each breeding pair was observed at least twice a week. At the time of banding, we collected DNA samples of adults and nestlings by puncturing the brachial vein and absorbing blood (max. 100 µl) with heparinized microcapillaries (permission number from the Cantonal Veterinary Office Zurich: 169/2001). Samples were either stored in microcapillaries directly or blown into APS-buffer (Arctander 1988) and stored at -20° C. We also collected dead nestlings and eggs that failed to hatch at -20°C for later DNA extraction.

Laboratory work

DNA from blood, unhatched eggs, and dead nestlings was extracted with the Qiagen "Biosprint 96 DNA Blood Kit". As characterized in Mayer et al. (2007) we used a set of 10 autosomal microsatellite loci (Emb 03, Emb 07, Emb 12, Emb 17, Emb 19, Emb 27, Emb 79, Emb 81, Emb 89, Emb 90, and Emb 112) and four additional z-linked microsatellite loci (Emb 79, Emb 84, Emb 107, and Emb 117) for parentage analyses. Polymerase chain reaction amplification was conducted as described in Mayer et al. (2007). Amplified fragments were visualized on an ABI PRISM 3730 Avant capillary sequencer. Allele sizes were determined in relation to an internal size standard (GeneScan-500LIZ) using GENEMAPPER version 3.7.

Parentage analysis

Based on the 10 autosomal microsatellite loci, parentage was determined in three steps using a likelihood-based approach in CERVUS 2.0 (Marshall et al. 1998). For all steps, the program screened candidate individuals and ranked them by the likelihood of being the

nestling's parent. First, maternity was determined for each nestling to check for egg dumping. This step included 232 broods. The genetic mother was identified in 208 broods, and no egg dumping was detected in these cases because the social mother always corresponded to the genetic mother. For the remaining 24 broods we did not have the genotype of the social mother. In the second step, paternity was assigned for the 208 nests using the mother as "known parent" in the analysis. The 10 autosomal microsatellite loci had a combined exclusionary power of 0.99984 for the first parent and 0.9999984 for the second parent. Finally, since we did not have the genotype of the social mother for 24 nests we determined paternity for those nests in a separate analysis without the genetic information of the social mother. Since in those cases the exclusionary power was reduced, multiple candidate fathers carrying common genotypes may have remained unexcluded. To increase the certainty of paternity we added information of the four sex-specific z-linked microsatellite loci and manually checked for congruence between offspring, their social fathers and the candidate father's genotypes. We did the same when nestlings did not amplify at all autosomal loci. Nestlings with bad DNA quality, i.e. that did not amplify at more than four autosomal loci, were excluded.

In cases where the social father, or the best candidate father, mismatched with the offspring genotype we checked the raw data for editing and typing errors. Seventeen nestlings mismatched at one locus with their potential genetic fathers. However, in all those cases no alternative candidate males had an almost similarly high likelihood of being the genetic father. When we compared those nestlings to their potential genetic fathers at the four z-linked loci, no mismatches could be detected. We therefore propose that the 17 mismatches arose from mutation. If we assume that highly polymorphic microsatellites mutate at the rate of 10^{-3} (Balloux & Lugon-Moulin 2002; Weber & Wong 1993) the number of observed mismatches is consistent with 16 mutations expected for our dataset (1171 individuals x 14 loci x 10^{-3}).

Dataset preparation

In total, our dataset contained 797 nestlings from 215 broods of 18 local populations from four years. There were no nestling data for one of the initial 19 local populations (Ambitzgi), because the single nest within this local population was lost to predation. We excluded some nests from the dataset before testing the effect of density on EPP for the following reasons. First, we excluded data of four nests (13 nestlings) because nestling DNA quality was too low to allow reliable paternity analysis. Two of those nests were the only

nests produced in local population Adletshausen. Therefore, the exclusion of those nests reduced the number of local populations to 17. Second, we excluded 19 broods (73 nestlings) from populations where only a single pair was breeding, because the density hypothesis requires that adults have the opportunity to encounter extra-pair mates. In our study area, breeding adults did not leave their local populations during the breeding season (Silvestri 2006). Therefore, in local populations with single breeding pairs, extra-pair matings would only have been possible if those local populations were visited by floater males. However, floaters can confound the effect of density on EPP. Three broods (13 nestlings) from local populations with single breeding pairs had unknown genetic fathers. This does not necessarily mean that they were sired by floater males since the genotype of their social fathers was unknown, too. Third, we excluded broods in cases where the genotype of the genetic and the social father was unknown (social father not captured). In those cases it was impossible to determine whether nestlings were sired by the social father or an unknown extra-pair male (8 broods with 31 nestlings). In one brood the social father's genotype was not known but for all nestlings the extra-pair father could be determined. We included this nest in the analysis. In 14 broods (50 nestlings) the genetic father of some of the nestlings (n = 33) could not be determined but the social fathers genotype was known. Since it was then possible to distinguish between within-pair young (WPY) and extra-pair young (EPY), those nests were included in the dataset. Finally, we excluded three broods (11 nestlings) from two polygynous males. Polygyny can have a strong influence on paternity (Rätti et al. 2001) since polygamous males have, but cannot guard, more than one female at the same time. Polygamous males may therefore more likely to be cuckolded in comparison to their socially monogamous neighbours (Birkhead & Møller 1992). At the same time, polygyny could enable later arriving females to choose an attractive male, making it unnecessary for those females to adjust their initial mate choice by pursuing extra-pair fertilizations. Thus, polygyny could also decrease the frequency of extra-pair fertilizations (Hasselquist et al. 1995).

Density estimation

As measures for local breeding density within populations, we calculated for each territory both the distance to the nearest reed bunting breeding territory in meters (hereafter referred to as 'nearest-neighbour distance'), and the number of territories within 170 m of the centre of the focal territory (hereafter 'number of neighbours'). Territory centres were defined as the geometric mean of all nests produced per territory per year. The radius of 170 m around a territory corresponds to the average distance between territory centres of extra-pair males

and the males they cuckolded within populations of our study area. While the nearest-neighbour distance only takes the distance to the next possible extra-pair partner into account, the number of territories within 170 m reflects the number of extra-pair mating opportunities within the neighbourhood of a focal territory. For comparisons among local populations, we calculated population density as the median nearest neighbour distance and the median number of neighbours, respectively, for each local population. Calculations of the nearest neighbour distance and the number of neighbours were done in ArcGIS 9.3.

Data analysis

To test for the relationship between density and extra-pair paternity, we used generalized linear mixed models with a logit link and binomial errors as implemented in the lmer procedure of the lme4 library, a contributed package to the open source statistical software R (R Development CoreTeam 2006). R carries out a weighted regression, using the number of nestlings per brood as weights, and the logit link function to ensure linearity.

We first tested for the effect of density on EPP within populations. This analysis comprised EPP data of broods of all local populations with annually more than one breeding pair collectively. The response variable was the EPP rate in a brood (i.e. EPY to total number of young per brood) and the model specification in R took into account varying brood sizes. Explanatory variables were the local breeding density (fixed effect) and the population-by-density interaction, population, year and female identity (random effects). Since the two measures for local breeding density, the nearest neighbour distance and the number of neighbours, were highly correlated ($r_s = -0.707$, $n = 181$, $p < 0.001$), we tested for their effects on EPP rate separately. The population-by-density interaction was included to test whether the relationship between density and EPP among broods of all local populations combined is also present within each local population. A random factor population was included in the model to estimate the variance in EPP that is generated due to specific characteristics of local populations. These characteristics, for example habitat structure, could potentially differ between local populations and thus confound a possible effect of density on variation in EPP. Year and female identity were included in the model to account for the variance in EPP levels generated by the effects of years and individual females' propensities to seek EPC. Female identity also accounted for dependencies arising from the use of data from multiple nests of the same female within and between seasons. We tested for significance of the interaction term and the random effects by running models with and without the interaction term and the random effects, respectively, and calculating likelihood ratio statistics.

To test for relations between population density and EPP at the level of local populations, we analysed models with EPP rate per local population as response variable and population density (fixed effect), population and year (random effects) as explanatory variables.We avoided pseudoreplication of broods by randomly selecting only a single brood for the 45 females that produced two or more broods within a given year and population.

Results

Paternity

Hundred-and-two broods (out of totally 181 broods from 13 local populations) contained at least one EPY (56.4%) and 248 nestlings (out of totally 669) were EPY (37.1 %). We identified 120 extra-pair fathers of which 23 had an unknown genotype (19.2 %). This does not necessarily mean that those males were floaters because it was not possible to band all paired males in all local populations, particularly in the three large ones (see Methods). For nine extra-pair fathers with known genotype the location of their territory was unknown. Three of them were banded after the year in which they sired extra-pair young, so that we were not able to locate their territory in the relevant year. The other six genotyped extra-pair fathers with unknown territories occurred in the three large populations, which supported more breeding pairs than we were able to monitor. Of the 88 extra-pair fathers, for which both genotype and territory location was known, 68.2 % were direct neighbours (adjacent territory), and 21.6 % were close neighbours (one territory in between) to the territories in which they sired EPY. Except for one male, that sired three nestlings within a brood of a neighbouring local population, extra-pair males exclusively sired EPY within local populations. Local populations occupied by single breeding pairs in a given year exclusively contained WPY (60 nestlings of 16 broods). Those nestlings were not included in the following analyses.

Relation between density and extra-pair paternity

The relationship between extra-pair paternity and local breeding density within populations was significant for both measures of local breeding density, negative for the nearest-neighbour distance and positive for the number of neighbours (Table 2). The density-by-population interaction was not significant (Table 2), indicating that there was a consistent relationship between EPP rate and local density within all local populations. The random factor year was significant for the model including the number of neighbours as estimate of local breeding density (Table 2). Variation in levels of EPP within populations was high, and

we found that female identity always explained a significant amount of the overall variance in EPP rate (Table 2).

To analyse the effect of density on EPP variation among local populations, we used a subset of the original dataset containing 500 nestlings from 135 broods out of 13 local populations, resulting in 34 population x year combinations. Population density measured as the median number of neighbours was positively related to EPP rate at the population level. When measrued as the median nearest-neighbour distance, population density was negatively related to EPP rate at the population level (Table 2, Fig. 1). Neither population nor year were significantly related to variance in EPP at the population level (Table 2).

Discussion

Local density at the level of the territory as well as population density explained variation in extra-pair paternity rate. Both results support the density hypothesis. Within populations, broods located in territories that had a lower distance to the nearest neighbouring territory and a higher number of neighbours also had increased levels of EPP (Table 2). Among populations, higher population density, measured as the median number of neighbours per population, was also positively associated to levels of EPP in the population. In addition, population density, measured as the median nearest-neighbour distance per population, was negatively related to the EPP rate per population.

Factors that confound tests of the density hypothesis

The among-population approach to test predictions of the density hypothesis has been surprisingly rarely applied. We are aware of only four studies that were explicitly designed to test the density hypothesis at the among-population level (Charmantier & Blondel 2003; Gibbs *et al.* 1990; Krokene & Lifjeld 2000; Yezerinac *et al.* 1999). Three of them supported the density hypothesis. In the only non-supportive study, Charmantier and Blondel (2003) compared a blue tit population on an island with one on the mainland that differed in EPP rate but not in density. This study obviously suffered from low statistical power since only two populations were compared. We tested the density hypothesis with data from 13 populations across four years. This is by far the largest dataset for which predictions of the density hypothesis have been tested among populations so far. Our study corroborates previous findings and emphasizes that density, if appropriately reflecting extra-pair mating opportunities, is an important ecological constraint explaining EPP variation among populations.

Comparison of EPP rates between many populations may impose problems arising from confounding factors such as habitat (Westneat & Mays 2005), climate (Bouwman & Komdeur 2006), or migration distance (Spottiswoode & Moller 2004). Previous studies were unable to account for these factors. In our study, differences among populations in migration distance or climatic conditions were very unlikely given our relatively small study area. Furthermore, we explicitly modelled the potential importance of unknown confounding factors, such as differences in habitat structure among local populations, by including population as a random factor, which however turned out to be non-significant in our analyses.

Most previous tests of the density hypothesis were done at a within-population level, and many of these tests suggested that density was a relevant factor explaining variation in EPP (Bjornstad & Lifjeld 1997; Charmantier & Perret 2004; Estep *et al.* 2005; Gowaty & Bridges 1991; Gray 1996; Hasselquist *et al.* 1995; Hoi & HoiLeitner 1997; Langefors *et al.* 1998; Lindstedt *et al.* 2007; Moller & Ninni 1998; Richardson & Burke 1999; Westneat & Sherman 1997). Our study corroborates these within-population analyses (Table 2). However, it is worth noting that within-population tests of the density hypothesis are also vulnerable to methodological or interpretation problems. For example, many of the studies that did not find support for the density hypothesis within populations suspected that their estimates of local breeding density did not reflect extra-pair mating opportunities, because a large proportion of EPCs occurred beyond the territory boundaries used to determine local breeding density (Dunn *et al.* 1994a; Moore *et al.* 1999; Reyer *et al.* 1997; Westneat & Mays 2005). In other studies, males were not territorial at least at the time when pursuing EPCs (Barber *et al.* 1996; Bollinger & Gavin 1991; Hill *et al.* 1994; Rätti *et al.* 2001), which decoupled breeding density from extra-pair mating opportunities. In all these cases where density estimates did not reflect extra-pair mating opportunities, the within-population approach is inappropriate because it overlooks that extra-pair mating opportunity, rather than density per se, is the mechanism underlying the hypothesis (Westneat et al. 1990). If breeding density is not correlated with extra-pair mating opportunities, then it is impossible to test the density hypothesis using local breeding density. In our study, the vast majority of males that sired EPY were close territorial breeding neighbours so that our measures of local density most likely reflected extra-pair mating opportunities. This was also the case in another study on reed buntings which, however, showed only a positive trend relating the proportion of EPP to local breeding densities (Bouwman & Komdeur 2006). Bouwman and Komdeur (2006) suggested that mate-guarding efforts increased with cuckoldry risk at increasing density

(Estep *et al.* 2005; Komdeur 2001), thereby masking the effect of density on EPP. This has also been assumed as explanation for the lacking relationship between density and EPP in other studies (Thusius *et al.* 2001; Westneat & Mays 2005). However, reed bunting mate-guarding efforts do not appear to vary with density (Marthinsen et al. 2005). As an alternative explanation for the insignificant relationship between EPP and local breeding density in their study, Bouwman and Komdeur (2006) suggested that local breeding density may have exceeded a certain threshold level resulting in sufficient extra-pair mating partners at all densities. A density threshold may also have obscured a relationship between density and EPP in other studies (Dunn *et al.* 1994b; Johannessen *et al.* 2005; Tarof *et al.* 1998). However, unambiguous support for the 'threshold hypothesis' initially proposed by Westneat et al. (1990) is still lacking. In our study, nearest neighbour distances varied between 10 and 270 meters, and the number of neighbours within 170 m of the focal territory between zero and 11. Bouwman and Komdeur (2006) did not report how local breeding densities varied in their population, so we can only speculate that variation in breeding density was sufficient for detecting a significant relationship between density and EPP in our study, but not in theirs. Another problem of previous within-population studies was the small sample size used (e.g., Chuang *et al.* 1999; Estep *et al.* 2005; e.g., Gullberg *et al.* 1992; Korpimaki *et al.* 1996; Lindstedt *et al.* 2007; Moore *et al.* 1999; Sundberg & Dixon 1996; Vaclav & Hoi 2002). Hence, many tests of the density hypothesis suffered from insufficient statistical power, and this greatly decreases confidence in not rejecting the null hypothesis of equal EPP rates at different densities (Moller & Ninni 1998). Of course, plenty of studies supporting the density hypothesis also had low sample sizes (Gibbs *et al.* 1990; Hill *et al.* 1994; Langefors *et al.* 1998). As noted previously, sample size was not of concern in our study, and estimates of EPP were of sufficiently high quality to justify the conclusions.

Which density estimate reflects extra-pair mating opportunities best?

Various density estimates have been used as proxies for extra-pair mating opportunities, but most have important shortcomings that confound tests of the density hypothesis. For example, the nearest-neighbour distance used in this study cannot distinguish between situations, where an individual has only one or multiple neighbours. Westneat et al. (1990) argued that the number of adjacent neighbours affects the likelihood of individuals seeking extra-pair mates, because it reflects extra-pair mating opportunities much better than the nearest neighbour distance. Charmatier and Perret (2004) show in their study on blue tits that the nearest neighbour distance had an effect on levels of EPP within broods when the

number of neighbours was low, but not when the number of neighbours was high. This finding reflects the limitation of the nearest neighbour distance as a reliable measure of extra-pair mating opportunities. However, the nearest neighbour distance has widely been used as estimate for local breeding density (for example, Bollinger & Gavin 1991; Gowaty & Bridges 1991; for example, Westneat & Sherman 1997) and results are mixed. In this study, the nearest neighbour distance was negatively related to local breeding and population density, but in all analyses this distance measure was less strongly related to levels of EPP than the number of neighbours (Table 2). The choice of density estimates in studies testing the density hypothesis should therefore be guided by careful consideration of the species' social system and spacing behaviour to avoid uninformative results.

Biological significance of density as a constraint to extra-pair paternity

The idea behind the density hypothesis is simple and strong. Density affects behaviour because it permits increasing interactions between individuals when proximity to or the number of neighbours increases (Westneat & Sherman 1997). Sexual interactions, like EPCs, seem to be especially sensitive to density since increased density provides better opportunities to decrease the costs of finding an additional mate (Westneat et al. 1990). A reduction in costs for seeking EPC may be one benefit of increased density to both males and females. In females, increased density may additionally allow improved assessment of potential extra-pair mates, if females engage in EPC only when they benefit from EPP. The number of potential extra-pair mates and hence the opportunities to engage in EPC with a high quality male likely increase with density leading to increased EPP levels.

The importance of density as a general underlying constraint to EPP might not be accepted if empirical evidence is simply assessed by counting the number of significant tests (see the critisism by Moller & Ninni 1998). Based on the number of studies published, evidence for the density hypothesis within or among populations is therefore usually cited as 'not consistent' (Griffith et al. 2002), 'equivocal' (Tarof et al. 1998) or 'contrasting' (Charmantier & Perret 2004). Contradictory evidence can easily be found in the literature, even within the same species (for example, red-winged blackbirds, see Gibbs *et al.* 1990; Westneat & Mays 2005), and this leads to the conclusion that the influence of density on EPP is not as consistent or strong (Neudorf 2004; Westneat & Stewart 2003) as initially envisioned (Birkhead 1979; Westneat *et al.* 1990). This conclusion may be premature, especially when considering that some studies have been cited as not supporting the density hypothesis, even though they did not apply any tests. No tests were applied in studies supposed to show either

no relationship (Ardern *et al.* 1997; Griffith *et al.* 1999; Gyllensten *et al.* 1990, as cited in Griffith et al. 2002) or even a negative relationship among populations of the same species (Hasselquist *et al.* 1995; Leisler *et al.* 2000 as cited in Griffith et al. 2002), and also in within-population analyses (Hill *et al.* 1994; Veiga & Boto 2000). One study measured breeding synchrony instead of density (Rowe & Weatherhead 2007). However, confounding factors and low test power of most studies that failed to support the density hypothesis put their results into perspective (see discussion above and for review Griffith et al. (2002)), but this has not been considered in discussions about the biological significance of density in explaining EPP variation (see the reviews of Neudorf 2004; see the reviews of Westneat & Stewart 2003).

Our data also illustrate a result that seems to be self-evident, but has been noted only once (Orell et al. 1997): extra-pair matings cannot occur where density is so low that no extra-pair opportunities are available. In our study, no EPP was detected in any population settled by only a single breeding pair in a given year (n = 16 population x year combinations). This example shows that density must indeed affect EPP.

Aside from local density, other factors appear to influence variation in EPP. For example, female identity always explained a significant amount of the variance in EPP in within-population analyses. Therefore, besides an underlying effect of density, other factors must explain additional variation in levels of EPP. For example, depending on the quality of their social mates, females may have different propensities to seek EPCs (Kempenaers et al. 1992), to obtain direct benefits like infertility insurance, or indirect benefits like good genes or an increase in heterozygosity of their offspring (Griffith *et al.* 2002; Westneat & Stewart 2003). These factors contribute to variation in EPP on top of the variation that is explained by density.

Conclusion

As the density hypothesis states, we show empirically that density, if reflecting extra-pair mating opportunities, explains variation in levels of EPP. Our approach to testing the density hypothesis includes within- and among-population analyses, which has previously been attempted only once and with a very small sample size (Krokene & Lifjeld 2000). Our data add to the list of studies that support the density hypothesis in within population analyses and also corroborate the findings of the few studies and meta-analyses supporting an effect of density on EPP rate at the population level within species (Moller & Ninni 1998; Westneat & Sherman 1997). Besides density, other factors contribute to variation in EPP depending on the

biology of a species. This may be the reason why the relationship between density and EPP has not been found in comparisons among species (Westneat & Sherman 1997). However, the between-species analysis of Westneat and Sherman (1997) was carried out with the nearest neighbour distance as density estimate, which may not accurately reflect extra-pair mating opportunities in some species (Charmantier & Perret 2004; Westneat *et al.* 1990). It may be worthwhile assessing the importance of density to variation in EPP rates among species using density measures that truly reflect extra-pair mating opportunities while taking confounding factors into account.

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Tables

Table 1 Location, coordinates, wetland size, old reed area (see methods in Pasinelli et al. 2008), and mean annual number of breeding pairs for the 19 reed bunting populations studied from 2002-2005.

Population	Coordinates	Size [ha]	Old reed area [ha]	Breeding pairs
Adletshausen *	47°16'/08°47'	4.2	0-0.022	0.25
Ambitzgi $	47°18'/08°48'	16.7	0-0.543	0.25
Bergli	47°16'/08°48'	5.6	0.300-0.356	1.75
Egelsee	47°15'/08°49'	16.3	0.059-0.559	2.25
Feldbach	47°14'/08°48'	2.7	0.383	2
Greifensee	47°19'/08°42'	44.1	0.972-1.382	12
Hellberg	47°18'/08°48'	1.9	0-0.096	0.5
Herrgass	47°16'/08°46'	2.4	0.181	0.25
Hopperen	47°22'/08°42'	8.7	0.244-0.376	0.75
Hüsli	47°16'/08°49'	14.0	0.133	2.25
Kämmoos	47°16'/08°50'	10.5	0.028-0.413	1.25
Lützelsee	47°16'/08°47'	54.7	1.314-1.812	12
Oberhöfler	47°18'/08°48'	38.5	0.201	0.5
Pfäffikersee	47°21'/08°47'	247.2	2.581	10.25
Sackried	47°21'/08°45'	5.7	0.522-0.881	1.25
Seeweidsee	47°16'/08°47'	5.2	0.364	1.5
Sulzbach	47°15'/08°45'	2.9	0.195	0.75
Uerzikon	47°15'/08°45'	10.9	0.478	3.75
Werrikon	47°22'/08°42'	13.0	0.626-0.853	2.75

* Excluded from analysis due to bad quality of nestling DNA samples. $ No genetic data available since nest was lost to predation.

Table 2 Summary of generalized linear mixed models testing the effect of density on extra-pair paternity within and among local reed bunting populations.

Effect	Within populations			Among populations		
	Estimate	test statistic	P	Estimate	test statistic	P
Nearest neighbour distance	-0.007 (0.003)	-2.375	0.018	-0.007 (0.003)	-2.097	0.036
Population	0.663 (0.425)	0.007	0.935	0.187 (0.432)	1.54	0.215
Population x Nearest neighbour distance	0.008 (0.002)	0.190	0.979			
Year	0.448 (0.349)	2.63	0.105	0.015 (0.123)	0.248	0.619
Female	2.681 (1.637)	81.01	< 0.001			
Number of neighbours	0.280 (0.061)	4.622	< 0.001	0.207 (0.044)	4.674	0.007
Population	0.000 (0.002)	0.000	1	0.000 (0.000)	0.000	1
Population x Number of neighbours	0.000 (0.001)	0.003	1			
Year	0.793 (1.708)	6.25	0.012	0.021 (0.146)	0.52	0.471
Female	2.205 (1.485)	58.47	< 0.001			

Fixed factors in within-population analyses were local breeding density estimated as the nearest-neigbour distance and the number of neighbours, respectively. Fixed factors in among-population analyses were population density estimated as the median nearest-neigbour distance and the median number of neighbours, respectively. In within-population analyses, random factors were population, the population-by-density interaction, year and female. In among-population analyses, random factors were population and year. For fixed effects, parameter estimates with (standard errors), z-values and p-values are given. For random effects, variance components with (standard deviation) as well as χ^2 values and p-values of likelihood-ratio tests are given.

Figure legends

Figure 1

Extra-pair paternity rate per population and year in relation to the median nearest neighbour distances per population and year. Each filled circle represents the median of the EPP rate within a site in a specific year. Lines (interquartile range) show the variation in EPP rates among territories within populations.

Figures

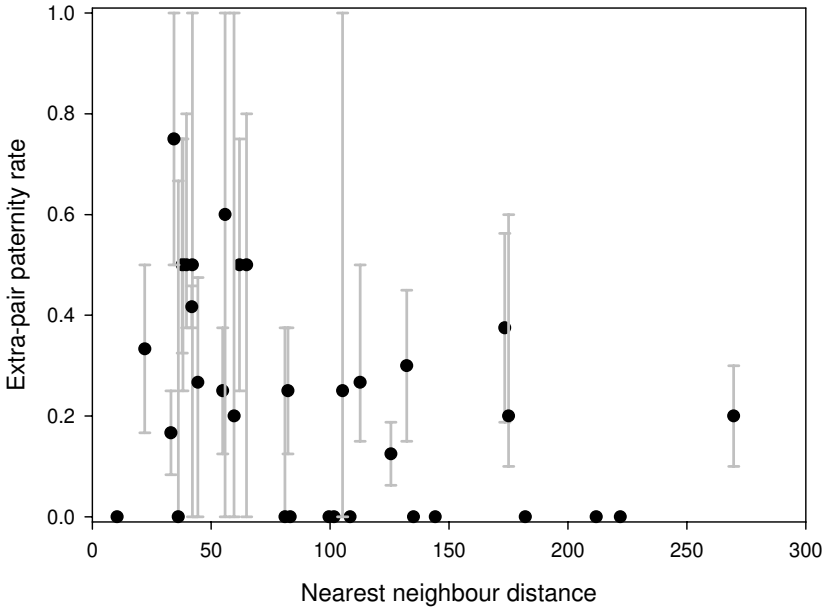

Fig.1

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Zoological Institute of the University of Zurich

CURRICULUM VITAE

Name	Mayer, Christian
Birth	02. Juni 1975
Citizenship	Schweppenhausen (Germany)

Education

2004 - 2009	PhD student in Zoology at the Univerity of Zurich
1996 - 2003	Diploma student in Biology at the University of Mainz (Germany) Title of the diploma thesis: "Phylogenie und Phylogeographie der Gattung *Pleurodeles*"
1994 - 1995	Training as emergancy medical technician and Civilian Service
1981 - 1994	Grundschule and Gymnasium with university-entrance diploma

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Mayer C, Schiegg K & Pasinelli G (2009): Patchy population structure in a short-distance migrant: evidence from genetic and demographic data. Molecular Ecology 18: 2353 - 2364.

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