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Endangered subspecies of the reed bunting (*Emberiza schoeniclus witherbyi* and *E. s. lusitanica*) in Iberian Peninsula have different genetic structures

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Running head: Different genetic structures in subspecies of reed bunting

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Abstract

In the Iberian Peninsula, populations of two subspecies of the reed bunting have become increasingly fragmented during the last decades when suitable habitats have been lost and/or the populations have gone extinct. Presently, both subspecies are endangered. We estimated the amount of genetic variation and population structure in order to define conservation units and management practices for these populations. We found that the subspecies *lusitanica* has clearly reduced genetic variation in nuclear and mitochondrial markers, has a drastically small effective population size and no genetic differentiation between populations. In contrast, the subspecies *witherbyi* is significantly structured, but the populations still hold large amounts of variation even though the effective population sizes are smaller than in the non-endangered subspecies *schoeniclus*. We suggest several management units for the Iberian populations. One unit includes subspecies *lusitanica* as a whole; the other three units are based on genetically differentiated populations of *witherbyi*. The most important genetic conservation measure in the case of *lusitanica* is to preserve the remaining habitats in order to at least maintain the present levels of gene flow. In the case of the three management units within *witherbyi*, the most urgent conservation measure is to improve the habitat quality to increase the population sizes.

Keywords: genetic diversity, effective population size, microsatellite, mtDNA, population structure

Zusammenfassung

Unterschiede in der genetischen Struktur von gefährdeten Unterarten der Rohrammer (*Emberiza schoeniclus witherbyi* und *E. s. lusitanica*) auf der Iberischen Halbinsel

Auf der Iberischen Halbinsel wurden die Populationen von zwei Rohrammer-Unterarten in den letzten Jahrzehnten, als geeignete Habitate verloren gingen und/oder Populationen ausstarben, zunehmend fragmentiert. Derzeitig sind beide Unterarten gefährdet. Wir schätzten die Höhe der genetischen Variation und die Populationsstruktur um daraus Einheiten für den Schutz und

Managementpraktiken für diese Populationen zu bestimmen. Wir fanden heraus, dass die Unterart *lusitanica* eine deutlich reduzierte genetische Variation in nukleären wie mitochondrialen Markern, eine dramatisch geringe effektive Populationsgröße und keine genetische Differenzierung zwischen Populationen aufweist. Im Gegensatz dazu ist die Unterart *witherbyi* deutlich strukturiert, aber die einzelnen Populationen beinhalten immer noch große Anteile der Gesamtvariation, wenngleich die effektiven Populationsgrößen kleiner sind als die der nicht gefährdeten Unterart *schoeniclus*. Wir schlagen mehrere Management-Einheiten für die Iberischen Populationen vor. Eine Einheit beinhaltet die Unterart *lusitanica* als Ganzes; die anderen drei Einheiten basieren auf den genetisch differenzierten Populationen der Unterart *witherbyi*. Die allerwichtigste Schutzmaßnahme aus genetischer Sicht im Falle der Unterart lusitanica ist die Bewahrung der verbleibenden Habitate um zumindest das derzeitige Maß an Genfluss zu erhalten. Im Falle der drei Management-Einheiten der Unterart *witherbyi* ist die dringlichste Schutzmaßnahme die Verbesserung der Habitate um die Populationsgrößen zu erhöhen.

Over the last twenty years there has been a debate on the importance of demographic and genetic processes in the chain of events leading to extinction. Lande (1988) emphasized the importance of demographic over genetic factors, but still sought for integration of both. Caughley (1994), while introducing the small-population paradigm and the declining-population paradigm argued that the small-population paradigm has contributed significantly to the theory of genetics and dynamics of small populations, but has so far been restricted largely to captive breeding; whereas the declining-population paradigm still is in need for more theory, and is really the one relevant to conservation. He stated that genetics often obscures the real issues, but also that this is not an argument of less conservation genetics, but more of it. In a large meta-analysis conducted by Spielman et al. (2004), in which 170 threatened taxa and their non-threatened taxonomic relatives were included, heterozygosity was found to be on the average 35% lower in the threatened taxa than the non-threatened relative taxa, and in 77% of pairwise comparisons the threatened taxa had lower heterozygosity. The authors argued that reduced genetic diversity indicates that the reproductive fitness is already compromised and extinction risk elevated. Even though Spielman et al. (2004) stated, that they were unable to determine whether genetic factors have contributed to the current threatened status of the taxa they studied, there are clear links between reduced genetic diversity and extinction risk. These links include the facts that 1) reduced genetic diversity reduces extinction times in changing environments, 2) change in heterozygosity between generations is a measure of inbreeding coefficient and related to population fitness and 3) inbreeding depression adversely affect the extinction risk.

Accepting the importance of genetic processes in conservation biology leads to a practical question of how to preserve the maximum genetic diversity in threatened species and how to define the units for management if resources available for the purpose are limited. Since Ryder (1986) presented the need to identify discrete populations within the range of a species, suggesting the use

of measures as genetic distances, multitude of concepts of such evolutionary significant units (ESUs) have been proposed. Later Waples (1991) proposed that an ESU should fill two criteria: it must be substantially reproductively isolated from other conspecific populations and it must represent an important component in the evolutionary legacy of the species. Moritz (1994) concretized the definition by stating that an ESU should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci. He also introduced the concept of management units (MUs) and defined them as populations with significant divergence of allele frequencies at nuclear or mitochondrial loci, regardless of the phylogenetic distinctiveness of alleles. Crandall et al. (2000) suggested that the rejection of ecological and genetic exchangeability forms the foundation of population distinctiveness and reciprocal monophyly should be omitted as a criterion because it is too restrictive. The discussion of defining conservation units has been going on with a goal to unify the concepts (e.g. Fraser and Bernatchez 2001) and with critics of different definitions (e.g. Patkeau 1999; Hey et al. 2003). The ultimate aim among conservation biologists is nevertheless the same: to find a common way to define the limits of distinct populations embracing as much of evolutionary history and adaptive potential as possible.

In this study, we aimed to estimate genetic distinctiveness and genetic diversity in endangered and still declining fragmented populations of the reed bunting (*Emberiza schoeniclus*) in the Iberian Peninsula. The reed bunting is a widely distributed Palaearctic passerine, common in most parts of its distribution range. At the western limits of the range, two subspecies of the reed bunting have been described for the Iberian Peninsula, *E. s. lusitanica* (Steinbacher 1930) in north-west and *E. s. witherbyi* (von Jordans 1923) in south-east, as well as the nominate subspecies *E. s. schoeniclus* (Linnaeus 1758), which breeds throughout north and central Europe and migrates to the south (including Iberian Peninsula) for wintering. The subspecies *E. s. lusitanica* is endemic to the Iberian Peninsula, while *E. s. witherbyi* is also found in southern France and in one wetland (Loukos) in Morocco. These subspecies are associated with wetlands (whereas *schoeniclus* inhabits a larger

variety of habitats especially in northern parts of the distribution range) and consequently their distribution is fragmented. Both *witherbyi* and *lusitanica* have drastically declined in numbers and range since the 70's, and the decline is still ongoing. For example, in 1995 the species was present in 74 Spanish wetlands, but ten years later was found in only in 35 wetlands. During 1995-2005, declines larger than 70% during were estimated for some of the regions from where census data is available. Both subspecies are considered as "Endangered" accordingly to UICN criteria (Atienza and Copete 2004). This fast decline in numbers and increased fragmentation has possibly reduced the genetic variation of the populations compared to populations still thriving. Therefore, we specifically aimed to estimate the amount of genetic variation in order to find if it is reduced and to define conservation units for the Iberian populations based on population distinctiveness using genetic measures. These results are discussed in relation to prospect of extinction of the populations and suggestions for conservation management are given.

Material and methods

Laboratory protocols

Samples from reed buntings were collected during 1995-2008 from northern Finland, Spain, Morocco and Portugal. Most of the samples were collected during the breeding season, but those of nominate subspecies *schoeniclus* from Spain were collected during winter and most of the samples from the delta of river Ebro during autumn, after the breeding season. In the autumn, both *schoeniclus* and *witherbyi* might co-occur in the delta of Ebro. Therefore the subspecies was identified according to morphometric measures (for example the bill of *witherbyi* is larger than the bill of *schoeniclus*; Byers et al. 1994). Samples were feather, blood (Spanish, Moroccan and Portuguese samples) or muscle tissue (Finnish samples). Iberian and Moroccan birds were released after measuring, ringing and sampling, for which the appropriate permits were obtained from the

respective authorities. Finnish samples were obtained from tissue collections of Zoological Museum of University of Oulu. Sample sizes and locations are shown in Table 1 and Fig. 1.

DNA was extracted from blood and muscle using the traditional phenol–chloroform extraction (Sambrook and Russell 2001) and from feathers using the lysis method described in Kvist et al. (2003). Six microsatellites *Esc3*, *Esc4*, *Esc6* (Hanotte et al. 1994), *Hru6* (Primmer et al. 1995), *Pdo5* (Griffith et al. 1999) and *Pocc6* (Bensch et al. 1997) were amplified in 10 µl reaction volume containing 50-100 ng of template DNA, 0.4 µM of each primer, 0.1 mM of each dNTP, 1 µl of 10 x PCR buffer and 0.06 units of DNA-polymerase (Biotools). The following PCR profile was used: 94°C for 5 min followed by 35 cycles of 94°C for 30s, annealing in 47-55°C for 30 s and 72°C for 30 s and a final extension in 72°C for 5 min. Annealing temperature for *Pocc6* was 53-55°C, for *Esc6*, *Hru6* and *Pdo5* 45-50°C and for *Esc3* and *Esc4* a touch down profile from 50-45°C was used. $MgCl_2$-concentrations varied from 2.0 mM for *Hru6* to 2.5 mM for *Esc 6*, *Pdo5* and *Pocc6* and 3.0 mM for *Esc3* and *Esc4*. The PCR products were run on ABI 3730 and alleles were scored with Genemapper v. 3.7.

About 770 bp long fragment of the mitochondrial control region was amplified with primers EmberizaL60 (5'CCCCAGCAACTTTTCTCCTG3') and PasseriformesH830 (5'GAATGGGGTCAAAGTGCATCAG3') using a PCR profile of 94°C for 5 min followed by 35 cycles of 94°C for 30s, 54°C for 30 s and 72°C for 30 s and a final extension in 72°C for 5 min. The amplification was performed in a 25 µl reaction volume containing about 150 ng of template DNA, 2 µM of each primer, 0.2 mM of each dNTP, 2.5 µl of 10 x PCR buffer (2mM $MgCl_2$) and 0.15 units of DNA-polymerase (Biotools) or 0.25 units of Dynazyme (Finnzymes). Sequencing of the PCR-products was performed with the same primers used for initial amplification with BigDye™ v. 3.1 Dye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacture and run with the ABI 3730 automatic sequencer.

Data analyses

Microsatellite data was checked for null-alleles and scoring errors using program Microchecker v. 2.2.3 (von Oosterhout et al. 2004). Existence of genetically structured populations was first tested with program Structure v. 2.2 (with no a priori information of the sampling locations, using 10 000 as the length of burnin periods and 100 000 MCMC replications, setting the number of populations (*K*) from one to twelve for two iterations and from one to five for additional two iterations, admixture model and correlated allele frequencies; Pritchard et al. 2000) and then by using Analysis of molecular variance (AMOVA) implemented in Arlequin v.3.11 (Excoffier et al. 1992). AMOVA was used also to find if there is genetic subdivision among the three studied subspecies by testing four different hierarchical structures; sampling sites were grouped into three groups according to defined subspecies and also each subspecies was combined with another into one group resulting in three possible combinations. Pairwise F_{ST} values between the sampling sites were calculated with Arlequin v.3.11, and the geographically close sites showing low and nonsignificant pairwise values were combined (one population for *lusitanica*, four for *witherbyi* corresponding to regions in Table 1). Assignment of each individual to the population of origin was performed also with Arlequin. Tests for linkage disequilibrium, Hardy–Weinberg equilibrium and calculation of F_{IS} were performed with Genepop v 4.0 (Raymond and Rousset 1995) and observed and expected heterozygosities were calculated with Arlequin. Effective population sizes were estimated using the linkage disequilibrium method implemented in program Ne-estimator v. 1.3 (Ovenden et al. 2007) for populations which had more than ten sampled individuals. Possible population bottlenecks were searched using the program Bottleneck v. 1.2.02 (Cornuet and Luikart 1996) and by calculating the Garza-Williamson index (*M*; Garza and Williamson 2001) implemented in Arlequin. Of the three options for mutation model for the microsatellites in program Bottleneck, we used the infinite allele model and the two-phase model with 70% of stepwise mutations. This program tests whether the heterozygosity is larger than expected given the number of alleles detected in each loci and checks

for a mode shift of allele frequency classes, which are both signs of a bottleneck. Garza-Williamson index compares the number of alleles of a locus to the allelic range. As a consequence of a bottleneck, the number of alleles decrease faster than the allelic range, leading to *M*-values lower than one.

Population structure of mitochondrial sequences was analyzed by calculating pairwise Φ_{ST} between the populations and performing the molecular variance analysis (AMOVA) as described for microsatellites. Φ_{ST} differs from F_{ST} by taking into account also genetic distances between haplotypes instead of only frequencies. Tamura-Nei's distance was used for these analyses, because it was the second best substitution model found by program MultiPhyl (Keane et al. 2007) after the HKY-model and included in program Arlequin (whereas HKY is not). Using the Tamura-Nei's distance instead of HKY is unlikely to influence the results, because the differences between the distance estimates are marginal. Nucleotide diversity, haplotype diversity and theta were estimated with DNAsp v. 4.10 (Rozas and Rozas 1999) for each population. Past changes in population size were studied by calculating Tajima's D, Fu's F and mismatch distributions as well as raggedness index and Ramos-Onsins and Rozas *R2* statistics with program DNAsp. In addition, maximum likelihood estimates of the growth rates (*g*) for the populations were calculated with program Lamarc v.2.1.2 (Kuhner 2006). This program estimates *g* based on exponential growth from $\theta_{(t)} = \theta_0 e^{-gt}$, where $\theta_{(t)}$ is θ at time t in the past and θ_0 is θ at present so that a positive value of g represents a growing population, and a negative value a shrinking population. The program was run using the 'likelihood mode' with 10 short chains and two final chains, discarding 1000 samples as burn-in and recording 10 000 genealogies. A parsimony network of the haplotypes was calculated with TCS (Clement et al. 2000).

Results

Microsatellites

Existence of possible null alleles was found in one locus (*Esc4*) in both, the Finnish and the Spanish *schoeniclus* populations, in one locus (*Esc3*) in Portuguese *lusitanica* population and in two loci (*Pocc6* and *Esc3*) in *witherbyi* population from Delta del Ebro. Otherwise no evidence of scoring errors, stuttering or null-alleles was found. As these loci were not constantly suspect of having null-alleles across different populations, it is likely that they rather show excess of homozygotes from other reasons than null-alleles and therefore all loci were used for analyses. No linkage was detected when tested across all the populations. When linkage was tested for each population separately, it was found in three populations; in Spanish *schoeniclus* population (*Pocc6* and *Hru6*), in Spanish *lusitanica* population (*Esc4* and *Hru6*) and in *witherbyi* population from Ebro (*Esc4* and *Hru6*).

Program Structure could not distinguish the populations nor subspecies (K=1, *Ln P* = -3232.4 – -3233.5, *var(LnP)* = 43.0 – 43.9, second best was for K=2, *LnP* -3252.3 – -3260.4, *var(LnP)* = 293.5 – 322.6). Pairwise F_{ST} values between sampling sites were low and nonsignificant between sampling sites of *shoeniclus* (F_{ST} = -0.0078) and *lusitanica* (F_{ST} = 0.00826) and between geographically close *witherbyi* populations from Villafranca (El Masegar included) and Daimiel (F_{ST} = 0.00878). These sampling sites were therefore combined in further analyses as *schoeniclus* (including sampling sites in Finland and Spain) *lusitanica* (sampling sites in Spain and Portugal) and Castilla La Mancha (including sampling sites of *witherbyi*, Villafranca, El Masegar and Daimiel in Spain). For these combined populations pairwise F_{ST} values (Table 2) were significant in all other comparisons except between *lusitanica* and *schoeniclus* and between the Mallorcan *witherbyi* population and other populations. Sample size from Marjal Pego-Oliva was small (n = 4, now the population is likely extinct), so the results concerning this population should be treated cautiously. Morocco was excluded from calculations of F_{ST} due to the small sample size. F_{ST} values estimated between the subspecies pairs were all significant, though relatively small (*schoeniclus-witherbyi*: 0.03381, *schoeniclus-lusitanica*: 0.02285 and *lusitanica-witherbyi*: 0.04288, all P-values < 0.05). Now also the pairwise F_{ST} value between *schoeniclus* and *lusitanica* became significant,

when *witherbyi* populations were grouped into subspecies. Results of molecular variance analyses using four different kinds of hierarchies are shown in Table 3. The analyses revealed that 2.68% of the total variance occurred between groups ($P < 0.05$) when the groups were formed according to subspecies. Also when *schoeniclus* and *lusitanica* were combined into one group, the variance between groups was almost as high (2.55%, $P < 0.05$). This is supported also by the pairwise F_{ST} values (Table 2).

The lowest heterozygosity values were found in *lusitanica* populations (*He* = 0.6543 and 0.6781) and in *witherbyi* populations from Marjal Pego-Oliva and Mallorca (*He* = 0.5631 and 0.6865, respectively), highest values (*He* = 0.7553 and 0.7602) a little surprisingly in *witherbyi* populations from Delta del Ebro and Castilla La Mancha (Table 4). Allele richness was the highest in *schoeniclus* (11.786) and the lowest in *lusitanica* (10.290). The value from *witherbyi* was close to that of *schoeniclus* (11.758). Differences between these values were non-significant (t-tests: *schoeniclus-lusitanica*, $P = 0.102$, *schoeniclus-witherbyi* $P = 0.493$, *witherbyi-lusitanica* $P = 0.073$). Estimates of the effective population sizes using the linkage-disequilibrium based method show the largest population sizes (87 and 133) for Spanish and Finnish *schoeniclus*, respectively, smaller estimates (21 and 53) for *witherbyi* (Delta del Ebro and Castilla La Mancha) and the smallest estimates (11 and 13) for Portuguese and Spanish *lusitanica* (Table 4). Program Bottleneck found no signs of a bottleneck in any of the populations (Wilcoxon test $P > 0.05$, no mode shifts), but the *M* ratio varied from 0.53 to 0.88. A ratio < 0.68 can, according to Garza and Williamson (2001), be assumed to indicate a reduction in size in any population analyzed for more than seven loci. With the six loci we analyzed, *M* ratios were less than 0.68 in two *witherbyi* populations, Marjal Pego-Oliva and Mallorca (0.58 and 0.53, respectively; however the sample sizes from these populations are small, which might affect the ratios). The ratios from Spanish *lusitanica* and *witherbyi* from Ebro were just slightly higher (0.682 and 0.685). Here again, these values need to be considered with some caution, because in addition to small sample sizes, the number of loci is smaller than

used by Garza and Williamson (2001). Assignment test classified almost all the individuals correctly to the population of origin. There were only seven exceptions, two individuals from Spanish *schoeniclus* population were assigned to Finnish *schoeniclus*, one bird from Spanish *lusitanica* was assigned to Portuguese *lusitanica* population, one to Spanish *schoeniclus*, one bird from Portuguese *lusitanica* was assigned to Spanish *schoeniclus* population, one individual from Castilla La Mancha was assigned to Spanish *lusitanica* and one to Portuguese *lusitanica*. All samples from Delta del Ebro, which were collected during autumn when overwintering individuals from northern populations of *schoeniclus* might have occurred at this site, were assigned to Ebro, so we concluded that this population sample is not likely to include misidentified individuals from *schoeniclus*.

Mitochondrial control region sequences

The 745 bp long alignment of the total of 125 sequences (GenBank accession numbers FJ794476-FJ794600) included 41 segregating nucleotide sites resulting to 38 haplotypes (haplotype diversity was 0.778). There were no double-peaks and no systematic differences that could be related to the tissue from which DNA was isolated, thus supporting the mitochondrial origin of the sequences. In addition, all obtained sequences overlapped in the central region and many were sequenced completely from both strands. Of the 36 sequences from *schoeniclus,* 23 haplotypes were found, in *lusitanica* there were only five haplotypes out of 48 sequences and in *witherbyi* 13 out of 41 sequences. One common haplotype (Es1; Fig. 2) was found from 56 individuals and it was represented in all the three subspecies. Other eight haplotypes (Es6, Es8, Es21, Es 36, Es59, Es64, Es76 and Es84; Fig. 2) were shared between two or more individuals and the remaining haplotypes were found only in one individual each (Fig 2, Appendix A). The parameters describing polymorphism within the subspecies (Table 4) showed low diversity in *lusitanica* and relatively high in *schoeniclus* and *witherbyi*. Theta was the highest in *schoeniclus* (0.00835), medium in

witherbyi (0.00525) and the lowest in *lusitanica* (0.00188). *Witherbyi* and *schoeniclus* had high nucleotide diversities (0.00312 and 0.00306, respectively), while it was low in *lusitanica* (0.00060). The nucleotide diversity in *lusitanica* was statistically highly different from that of *schoeniclus* and *witherbyi* (both t-tests resulted in $P < 0.0001$).

Pairwise Φ_{ST} values estimated between the subspecies pairs were again all significant (*schoeniclus-witherbyi* = 0.05331, *schoeniclus-lusitanica* = 0.04318 and *lusitanica-witherbyi* = 0.13968, all *P*-values < 0.05). Estimates between *schoeniclus* and the two other subspecies were quite small, but the estimate between *witherbyi* and *lusitanica* relatively large. There was no differentiation between sampling sites of *schoeniclus* (Φ_{ST} = -0.00527, *NS*) and *lusitanica* (Φ_{ST} = -0.01437, *NS*). Also no differentiation was detected between *witherbyi* samples from Villafranca and Daimiel (Φ_{ST} = 0.00173, *NS*). Therefore these sampling sites were combined as was done with microsatellite data into *schoeniclus*, *lusitanica* and *witherbyi* of Castilla La Mancha (from El Masegar, the third sampling site from this area, we did not succeed to sequence any samples). The Castilla La Mancha population differed significantly from the other *witherbyi* populations. In addition, significant differentiation was found between populations from Mallorca and Delta del Ebro and between populations from Marjal Pego-Oliva and Mallorca and Delta del Ebro (Table 5). The population of Marjal Pego-Oliva is represented by just four samples and therefore the pairwise Φ_{ST} -values do not necessarily represent reliably the true values. Morocco is again excluded from estimating the pairwise Φ_{ST}s. Hierarchical AMOVA showed that the among group variance was the largest and significant when grouping was formed based on the three subspecies or by grouping *schoeniclus* and *lusitanica* together (Table 3), i.e. *witherbyi* is differentiated from the two other subspecies (though variance among sampling sites is higher than among groups). Also differentiation between *lusitanica* and *schoeniclus* is supported especially by the pairwise Φ_{ST}-values.

Mismatch distributions from all the sequences combined followed closely the expected distribution for 'recent' population growth/decline. θ initial (θ before the population size change) and τ (time of the size change in mutational time $2ut$, where u is the mutation rate and t is time in generations) describing the shape and mean of the distribution were 0.671 and 0.956, respectively, (0.969 and 1.239 for *schoeniclus*, 0.360 and 0.072 for *lusitanica* and 0.000 and 2.251 for *witherbyi*). θ final was 1000 for all. Raggedness statistics was 0.0384 (P = 0.058) and Ramos-Onsins and Rozas *R2* statistics 0.0201 (P < 0.001) for the combined set of sequences. The mismatch distributions and diversity values of the subspecies compared to each other and to the combined values show that the peak of the mismatch distribution and all the diversity values are clearly the lowest in *lusitanica*, especially in the Spanish population, indicating a loss of haplotypes and diversity. Growth rates (g) estimated with program Lamarc were very large for *schoeniclus* (2 315, with 95% confidence intervals of 1727 – 2879), large also for *witherbyi* (819; 95% CI 521 – 1086) and negative for *lusitanica* (-1 413; 95% CI -2408 – -684).

Discussion

Diversity within populations

Mitochondrial DNA sequences showed that genetic diversity was significantly reduced in the Iberian subspecies of the reed bunting, *E. schoeniclus lusitanica*. Some indications of reduction in diversity could also be seen in microsatellites (allelic richness was the lowest), but this was not significant. Furthermore, the Spanish *lusitanica* population had reduced heterozygosity values and both, the Portuguese and Spanish populations had extremely low effective population sizes (N_e 10.9 and 13.4, respectively). The differences in the magnitude of the reduction in genetic diversity in the two marker systems are likely due to the different effective population sizes of the markers. The decrease of the census population sizes has affected the mitochondrial sequence diversity faster, because the effective size and therefore also the coalescent time of mitochondrial markers is only

one quarter of the nuclear markers, and thus the genetic drift may act four times stronger, rapidly removing rare haplotypes from the small populations. Usually, the extremely fast mutation rate of the microsatellite markers is thought to result in easier detection of very recent demographic events than would be possible with mitochondrial markers (see Zink and Barrowclough 2008). But in our study, especially in the case of the *lusitanica* populations, drift has reduced much more variation in mitochondrial markers than in microsatellites.

The Iberian subspecies *witherbyi*, on the other hand, did not show as strong reduction of genetic diversity as expected based on the fast decrease of the census sizes. The three populations with adequate sample sizes (Delta del Ebro, Castilla La Mancha and Mallorca) had mitochondrial nucleotide diversity and nuclear heterozygosity values that were at the same level as in the nominate *schoeniclus* populations, even though for Delta del Ebro and Mallorca the decline of the population has been above 70% in the last decade (no census data available for Castilla La Mancha but the population probably is more or less stable). Only the number of alleles and the allelic richness in four of the six studied microsatellite loci (*Esc3*, *Esc4*, *Esc6* and *Pdo5*, Appendix B), haplotype diversities and theta values were slightly lowered. It is possible that the decline is still so recent, that drift has just begun to reduce the number of rare haplotypes and alleles, but this is not yet detectable by different bottleneck tests or diversity values. In the case of the Castilla La Mancha population, it is also possible that even though *F*-statistics did not find differences between the sampling sites within this area, we had sampled individual from distinct populations, which might have increased the genetic variation via Wahlund's effect. Some additional evidence of decreased census size affecting genetic patterns in *witherbyi* was obtained from low Garza-Williamson indexes (Delta del Ebro and Mallorca populations) and relatively small effective population sizes (20.6 for Delta del Ebro and 53.4 for Castilla La Mancha).

Overall, the heterozygosity values were somewhat lower than previously reported in a study by Matessi (1999), where four loci were used (three of those were same as here). One of our study

populations, the Delta del Ebro population, was also included in Matessi (1999), and then the observed and expected heterozygosities for that population were 0.781 and 0.823, respectively (n = 16). It is possible that heterozygosity has decreased during the years between the sampling for Matessi's and for our study. Our first sampling period from this population was 1995, and including only those individuals resulted in slightly higher value of expected heterozygosity (0.7874), which anyhow was still lower than the value obtained by Matessi (1999). It is therefore more likely that our values were in general lower due to the used marker set or small sample size. In a Swiss population, belonging to the nominate subspecies, heterozygosity in eleven autosomal microsatellite loci varied from 0.756 to 0.933 (n = 45; Mayer et al. 2008).

Differentiation of subspecies and populations

The number of subspecies in the reed bunting varies from 30 to 15 depending on authors and these subspecies are grouped into two to four groups (Byers et al. 1994; Cramp and Perrins 1994). The subspecies are designated largely based on bill size and plumage colour. Genetic differentiation between the subspecies groups, subspecies or populations of the reed bunting has not been studied in detail and only a couple of subspecies have been included in published studies. Graputto et al. (1998) have shown that subspecies *intermedia* of southern, thick-billed *pyrrhuloides*-group and *schoeniclus* of the northern thin-billed *schoeniclus*-group are slightly genetically differentiated in nuclear loci (F_{ST} from four microsatellite loci was 0.0444), but not in mitochondrial DNA. Our microsatellite data resulted in very similar F_{ST} values (0.0229-0.0429) between the subspecies, but also showed significant differentiation with mitochondrial data (pairwise Φ_{ST} values 0.0432-0.1397). This difference may be explained by the highly variable mitochondrial control region sequences used here, which are more suitable for detection of genetic structures within species than the more conservative mitochondrial cytochrome b and ND5 sequences used by Graputto et al.

(1998). Also, hierarchical molecular variance analyses from both marker sets supported some genetic differentiation between the three subspecies.

Genetic differentiation among populations within subspecies was not evident in *schoeniclus* or *lusitanica*, whereas among populations of *witherbyi*, the differentiation was surprisingly large in many cases. Within *witherbyi*, pairwise F_{ST} values were high and significant (range 0.0381-0.1402) between all populations except comparisons to Mallorca. In addition, Φ_{ST}-values were significant (range 0.1299-0.2861) in all except some comparisons involving Marjal Pego-Oliva, which could be just due to the small sample size. Notably, many of the values within *witherbyi* were much higher than values estimated between subspecies. Even though the sample sizes were not large for some of the populations, it seems that the *witherbyi* populations are more differentiated from each other than *lusitanica* populations or the migrant *schoeniclus* populations. Unfortunately, estimates of pairwise population differentiation presented by Graputto et al. (1998) and Matessi (1999) were calculated using coancestry coefficients or Nei's genetic distances, and cannot be directly compared with our results. However, estimates of F_{ST} were given among populations of *schoeniclus* (0.0361) and among populations of *intermedia* (0.0277), which are clearly higher than our estimates for *schoeniclus* and *lusitanica*, but much lower than our estimates for *witherbyi*.

The difference in the magnitude of genetic structure reflects the different amounts of gene flow among populations within the subspecies. Both *lusitanica* and *witherbyi* have inhabited a larger amount of wetlands in the past, but now have gone extinct especially from small wetlands. It is possible, that in *lusitanica* the gene flow, i.e. dispersal between the fragmented habitats, is more effective, aiding recolonizations after local extinctions (following more or less the metapopulation model). On the other hand, the geographical distances between populations of *lusitanica* are shorter than distances between populations of *witherbyi* because the current distribution area of *lusitanica* is much smaller than that of *witherbyi*. This might be the reason why gene flow seems to be more effective in *lusitanica*. In any case, differentiation between populations of *witherbyi* is stronger than

in *lusitanica*, suggesting low amounts of gene flow. Unfortunately, this might indicate that the now extinct populations are lost for good.

Implications for conservation

Habitat loss was probably the main cause of decline in the 1970's and 1980's. However, already in the last decade, most of the populations were located within protected areas and therefore the loss of wetlands cannot account for the continuing decline. Belda et al. (2008) suggested that changes associated to reed and water management in wetlands are also an important cause for the decline of the species. Traditional activities, such as grazing, cutting, etc., have been abandoned or banned in these protected areas, and the decline or extinction of the reed bunting in these areas has been recorded to follow those changes in management (unpublished data). Most of the management practices have been devoted to favor other species, such as endangered ducks or egrets, without knowing how the consequences affect other bird species, such as several endangered passerines like the species studied here. Therefore, there is an urgent need to undertake studies on habitat requirements of *lusitanica* and *witherbyi* and to understand how reed management affects demographic parameters. As for now, it could be a promising idea to allow or even encourage the traditional use of the wetlands, at least in some parts, for getting a more diverse habitat, which would fill the requirements for a variety of species.

In the light of the estimated genetic differentiation, there are no evolutionary significant units in Iberian reed buntings, but we suggest several management units for Iberian reed bunting populations. One unit includes subspecies *lusitanica* as a whole. *Lusitanica* is differentiated from other subspecies, has reduced genetic variation especially in mitochondrial markers, a drastically small effective population size and a negative growth rate. The Salreu population in Portugal is presently estimated to be around 350-400 breeding pairs, while for Galicia, Spain, the estimate is around 50-60 breeding pairs, distributed in 14-15 wetlands. Given that there is no differentiation

between populations and some of the populations hold only a few breeding pairs, it is quite likely that those small populations receive immigrants from the 'large' Salreu population and possibly also from other smaller populations. If this is the case, the most important genetic conservation measure would be to increase or at least maintain the present levels of gene flow. To achieve this aim, the remaining habitat network needs to be preserved.

Other suggested management units are *witherbyi* populations from Delta del Ebro, Castilla La Mancha and Mallorca. These populations show some genetic differentiation in one or both markers and therefore are likely to hold variation not present in other *witherbyi* populations. The population from Marjal Pego-Oliva fulfils also these criteria, but in this case the sample size is too low to make any suggestions (and the population might actually be already extinct). Even with a moderate amount of gene flow in general, recolonizations of small and geographically isolated habitats located far from each other are highly unlikely. At present, the remaining census sizes for Castilla La Mancha (in Daimiel about 100 pairs) and Delta del Ebro (50-100 pairs) are already alarmingly low. The most urgent conservation measures should therefore be guided to maintain these two mainland populations in addition to the even more threatened population in Mallorca. The only way to do this is to offer enough of proper habitats to help to increase the population sizes. In other words, there is an uttermost need to understand the habitat requirements and demography of the endangered Iberian subspecies before it is too late.

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Appendix A Haplotype distribution. Abbreviations for population names: schF = *E.s..schoeniclus*, Finland, schS = *E. s. schoeniclus* Spain, lusS = *E. s. lusitanica*, Spain, lusP = *E. s. lusitanica*, Portugal, witEb = *E. s. witherbyi*, Delta del Ebro, witVFr = *E. s. witherbyi*, Villafranca, Castilla La Mancha, witDa = *E. s. witherbyi* Daimiel, Castilla la Manca, witMPO = *E. s. witherbyi*, Marjal Pego-Oliva, witMa = *E. s. witherbyi*, Mallorca, witMo = *E. s. witherbyi*, Morocco

	Population									
Haplotype	schF	schS	lusS	lusP	witEb	witVFr	witDa	witMPO	witMa	witMo
Es1	5	7	20	19	1	1	3			
Es2	1									
Es4	1									
Es7	1									
Es8	1	1								
Es10	1									
Es12	1									
Es13	1									
Es14	1									
Es36		2								
Es39		1								
Es40		1								
Es41		1								
Es42		1								
Es43		1								
Es44		1								
Es48		1								
Es49		1								

Es50		1								
Es51		1								
Es53		1								
Es55		1								
Es21			1	5						
Es123				1						
Es133				1						
Es136				1						
Es58					1					
Es59					2			1		
Es64					2	1	1	2	4	
Es6	1					3	5			2
Es72						1				
Es116						1				
Es142						1				
Es76								1	3	
Es79									1	
Es84							2			
Es90							1			
Es93							1			
Total	14	22	21	27	6	8	13	4	8	2

Appendix B Number of alleles (*#A*) and allelic richness (*R*) for each locus and study population. See abbreviations of population names from Appendix A

	Population									
Locus	schF	schS	lusS	lusP	witEb	witVFr	witDa	witMPO	witMa	witMo
	# A R	*# A R*	*# A R*	*# A R*	*# A R*	*# A R*	*# A R*	*# A R*	*# A R*	*# A R*
Escmu3	13 1.913	14 1.93	7 1.782	9 1.780	9 1.895	7 1.804	12 1.850	3 1.600	7 1.894	2 1.667
Escmu4	14 1.924	13 1.910	15 1.888	11 1.890	9 1.908	8 1.900	12 1.900	3 1.679	7 1.909	3 1.833
Escmu6	9 1.841	11 1.869	6 1.754	10 1.872	6 1.817	6 1.850	7 1.803	3 1.600	5 1.703	2 1.667
Pdo5	17 1.952	19 1.947	14 1.845	16 1.917	10 1.918	8 1.908	13 1.885	4 1.786	8 1.894	2 1.667
Pocc6	2 1.370	2 1.359	4 1.418	4 1.572	4 1.592	5 1.549	5 1.346	3 1.464	2 1.167	2 2.000
HrU6	2 1.067	5 1.207	4 1.241	2 1.037	4 1.403	5 1.621	7 1.677	2 1.250	4 1.396	2 1.667
Total	57 1.678	64 1.704	50 1.655	52 1.678	42 1.756	39 1.772	56 1.744	18 1.563	33 1.661	13 1.750

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Figure legends

Fig. 1 Sampling sites of the Iberian reed bunting

Fig. 2 A parsimony network from the mitochondrial control region sequences. Sizes of the circles are proportional to the number of haplotypes found and shadings infer the origins of the individuals possessing the haplotypes. Each connecting bar represents one substitution

Table 1 Sampling sites and sizes of the reed buntings

Subspecies	Year	Wetland	Locality	Region	*n* wetland	*n* region
schoeniclus	2002-5	–	Oulu	Northern Finland		15
schoeniclus	2005-6	Marjal Pego-Oliva	Oliva	Valencia, Spain	23	23
lusitanica	2006-8	Carrizales del Ulla	Dodro	Galicia, Spain	13	
lusitanica	2006-8	Estuario del Miño	A Guarda	Galicia, Spain	4	23
lusitanica	2006	Lestimoño	Ponteceso	Galicia, Spain	6	
lusitanica	2003-8	Salreu	Salreu	Estarreja, Portugal	29	29
witherbyi	1995, 2006	Delta del Ebro	Delta del Ebro	Cataluña, Spain	11	11
witherbyi	2006-7	Lagunas de Villafranca de los Caballeros	Villafranca de los Caballeros	Castilla La Mancha, Spain	12	34
witherbyi	2006-7	Tablas Daimiel	Daimiel	Castilla La Mancha, Spain	19	
witherbyi	2007	El Masegar	Quero	Castilla La Mancha, Spain	3	
witherbyi	2006-7	Marjal Pego-Oliva	Oliva	Valencia, Spain	4	4
witherbyi	2006-8	S'Albufera	Mallorca	Baleares, Spain	12	12
witherbyi	2008	Marismas de Loukos	Larache	Morocco	2	2

Table 2 Pairwise F_{ST} values from microsatellite data. Values in bold are significant with $P < 0.05$

	E.s.schoeniclus	*E.s.lusitanica*	*E.s.witherbyi* Ebro	*E.s.witherbyi* Castilla La Mancha	*E.s.witherbyi* Marjal Pego-Oliva
E.s.lusitanica	0.02285				
E.s.witherbyi Ebro	**0.03335**	**0.04644**			
E.s.witherbyi Castilla La Mancha	**0.04458**	**0.05859**	**0.03806**		
E.s.witherbyi Marjal Pego-Oliva	**0.09214**	**0.10788**	**0.09113**	**0.09564**	
E.s.witherbyi Mallorca	0.00211	0.00882	-0.01530	0.00464	0.03027

Table 3 AMOVA results. Sampling sites: *E. s. schoeniclus* Finland, *E. s. schoeniclus* Spain, *E. s. lusitanica* Portugal, *E. s. lusitanica* Spain, *E. s. witherbyi* Delta del Ebro, *E. s. witherbyi* Castilla La Mancha, *E. s. witherbyi* Marjal Pego-Oliva, *E. s. witherbyi* Mallorca, *E. s. witherbyi* Morocco

Marker	Hierarchy	Variance components	Percentage of variation	*P*	F_{ST}
Microsatellites					
	3 groups: *schoeniclus*, *lusitanica* and *witherbyi* 9 sampling sites	among groups	2.68	0.0059	0.0433
		among sites within groups	1.65	< 0.001	
		within sites	95.67	< 0.001	
	2 groups: *schoeniclus* and *lusitanica* combined 9 sampling sites	among groups	2.55	0.0117	0.0484
		among sites within groups	2.28	< 0.001	
		within sites	95.17	< 0.001	
	2 groups: *schoeniclus* and *witherbyi* combined 9 sampling sites	among groups	1.52	0.1750	0.0443
		among sites within groups	2.91	< 0.001	
		within sites	95.57	< 0.001	
	2 groups: *lusitanica* and *witherbyi* combined 9 sampling sites	among groups	0.66	0.3851	0.0410
		among sites within groups	3.44	< 0.001	
		within sites	95.90	< 0.001	
mtDNA					
	3 groups: *schoeniclus*, *lusitanica* and *witherbyi* 9 sampling sites	among groups	3.11	0.1701	0.1399
		among sites within groups	10.89	0.0449	
		within sites	86.01	< 0.001	
	2 groups: *schoeniclus* and *lusitanica* combined 9 sampling sites	among groups	6.92	0.0176	0.1628
		among sites within groups	9.36	0.0010	
		within sites	83.73	< 0.001	

	2 groups: *schoeniclus* and *witherbyi* combined 9 sampling sites	among groups	1.03	0.2659	0.1378
		among sites within groups	12.77	< 0.001	
		within sites	86.2	< 0.001	
	2 groups: *lusitanica* and *witherbyi* combined 9 sampling sites	among groups	-2.96	0.5621	0.1209
		among sites within groups	15.5	< 0.001	
		within sites	87.91	< 0.001	

Table 4 Polymorphism measures from the study populations. From left to right: sample sizes used in microsatellite analyses (*n*), observed and expected heterozygosities (*Ho*, *He*), inbreeding coefficient (F_{IS}), N_e estimates using linkage disequilibrium method (95% confidence interval), sample sizes and haplotype numbers from mitochondrial data (n/#hapl), nucleotide diversity (π), number of segregating sites (θ) haplotype diversity ($\hat{h}$), Fu's F and Tajima's D and their significance

	n	*Ho*	*He*	F_{IS}	N_e (95%CI)	*n/#hapl*	π	θ	$\hat{h}$	Fu's F	Tajima's D
E. s. schoeniclus	38	0.6655	0.6923	0.0412	–	36/21	0.00306	0.00835	0.871	–26.7609 *P* < 0.001	–2.1842 *P* < 0.01
Finland	15	0.6667	0.6778	0.0170	132.8 (43–inf)	14/10	0.00423	0.00784	0.890	–14.0699 *P* < 0.001	–1.9079 *P* < 0.05
Spain	23	0.6625	0.7037	0.0607	87 (43–1097)	22/14	0.00234	0.00570	0.874	–27.3521 *P* < 0.001	–2.1224 *P* < 0.01
E. s. lusitanica	52	0.6632	0.6850	0.0222	–	48/5	0.00060	0.00188	0.330	–inf *P* < 0.001	–1.7474 *P* < 0.01
Spain	23	0.6006	0.6543	0.0445	13.4 (1–19)	21/2	0.00013	0.00038	0.095	–inf *P* < 0.001	–1.1636 *P* = *NS*
Portugal	29	0.6951	0.6781	–0.0242	10.9 (9–13)	27/5	0.00095	0.00217	0.484	–inf *P* < 0.001	–1.6435 *P* < 0.05
E. s. witherbyi	57	0.7028	0.7584	0.0751	–	41/13	0.00312	0.00525	0.866	–26.8519 *P* < 0.001	–1.2745 *P* = *NS*
Ebro	11	0.6368	0.7553	0.1688	20.6 (11–99)	6/4	0.00314	0.00303	0.867	–4.0049 *P* < 0.01	0.1965 *P* = *NS*
Castilla La Mancha	31	0.7398	0.7602	0.0288	53.4 (34–110)	21/9	0.00236	0.00388	0.829	–27.4862 *P* < 0.001	–1.3347 *P* = *NS*
Marjal Pego-Oliva	4	0.5972	0.5631	–0.0685	–	4/3	0.00439	0.00456	0.833	–1.1571 *P* = *NS*	–0.1345 *P* = *NS*
Mallorca	9	0.6865	0.6604	–0.0491	–	8/3	0.00331	0.00268	0.679	–6.5008 *P* < 0.001	1.0923 *P* = *NS*

Table 5 Pairwise Φ_{ST}-values from mitochondrial sequence data. Values in bold are significant with $P < 0.05$

	E.s.schoeniclus	*E.s.lusitanica*	*E.s.witherbyi* Ebro	*E.s.witherbyi* Cast. la Mancha	*E.s.witherbyi* Marjal Pego-Oliva
E.s.lusitanica	**0.04318**				
E.s.witherbyi	0.04669	**0.3637**			
Ebro					
E.s.witherbyi	**0.08165**	**0.2168**	**0.1299**		
Castilla La Mancha					
E.s.witherbyi	0.05345	**0.4545**	-0.1510	**0.1465**	
Marjal Pego-Oliva					
E.s.witherbyi	**0.19073**	**0.5365**	**0.1250**	**0.2775**	-0.1236
Mallorca					