

Genetic variation and bill size dimorphism in a passerine bird, the reed bunting *Emberiza schoeniclus*

A. GRAPPUTO,* A. PILASTRO and G. MARIN

Dipartimento di Biologia, Università di Padova, via Colombo 3, I-35121 Padova, Italy

Abstract

In passerine birds morphological differentiation in bill size within species is not commonly observed. Bill size is usually associated with a trophic niche, and strong differences in it may reflect the process of genetic differentiation and, possibly, speciation. We used both mitochondrial DNA (mtDNA) and nuclear microsatellites to study genetic variation between two subspecies of reed bunting, *Emberiza schoeniclus schoeniclus* and *E.s. intermedia*, along their distributional boundary in western Europe. These two subspecies are characterized by a high dimorphism in bill size and, although breeding populations of the two subspecies are found very close to each other in northern Italy, apparently no interbreeding occurs. The observed morphological pattern between the two subspecies may be maintained by geographically varying selective forces or, alternatively, may be the result of a long geographical separation followed by a secondary contact. MtDNA sequences of cytochrome *b* and ND5 (515 bp) showed little variation and did not discriminate between the two subspecies, indicating a divergence time of less than 500 000 years. The analysis of four microsatellite loci suggested a clear, although weak, degree of genetic differentiation in the large- and small-billed populations, as indicated by F_{ST} and R_{ST} values and genetic distances. The correlation between bill size and genetic distance between populations remained significant after accounting for the geographical distances between sampling localities. Altogether, these results indicate a very recent genetic differentiation between the two bill morphs and suggest that a strong selection for large bills in the southern part of the breeding range is probably involved in maintaining the geographical differentiation of this species.

Keywords: *Emberiza schoeniclus*, gene flow, microsatellite loci, morphology, mtDNA sequences, population genetics

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Introduction

High polymorphism in bill size is very unusual in passerine birds. Notable exceptions are Darwin's finches (*Geospiza*), a classical and well studied case of inter- and intraspecific variability in bill size among related birds inhabiting different islands of the Galapagos archipelago (Grant 1986). Such polymorphism can be associated with very specialized feeding, as in the case of *Geospiza difficilis*, which feeds on the blood of sea birds. Intraspecific variation in bill size is usually lower than interspecific variation in Darwin's finches, although bill depth can vary

intraspecifically in relation to climatic conditions that alter the production of different types of seeds. In this case, directional selection causes bill size to shift in subsequent years with the abundance of large and hard seeds, which favours individuals with stronger bills.

Another interesting case is the African finch *Pyrenestes ostrygnus*, which has two sympatric forms distinguishable by the use of different seed types (Smith 1987). This appears to be a typical case of disruptive selection causing the appearance of two bill morphs (genetically controlled by a single locus), which reduces intraspecific competition for food by extending the trophic niche of the species (Smith 1990). In both *Geospiza* and *Pyrenestes*, differences in bill size are associated with differences in diet, and larger bills allow the exploitation of larger and harder seeds.

Correspondence: A. Grapputo. *Present address: Centre for Biodiversity and Conservation Biology, Royal Ontario Museum, 100 Queen's Park, Toronto, Ontario, Canada. M5S 2C6. Fax: +1 416 586 5553; E-mail: alessand@rom.on.ca

The reed bunting (*Emberiza schoeniclus*), a passerine widely distributed in Europe and Asia, represents a third case of considerable intraspecific polymorphism in bill size. This species occurs in populations (subspecies) characterized by large variances in bill depth, with large-billed birds having a bill depth of about 8 mm, almost twice as deep as that observed in small-billed individuals (Cramp & Perrins 1994). Historically, more than 30 subspecies have been described, based largely on bill size and plumage colouration. These subspecies have been grouped into two (Cramp & Perrins 1994), three (Blümel 1982) or four groups (Byers *et al.* 1995). In all cases, there seems to exist a clear differentiation between northern, migratory populations, with small bills, and southern, sedentary populations, with large, deep bills (Cramp & Perrins 1994). Although this aspect has been poorly investigated, bill size variation appears to be associated with differences in feeding ecology. In particular, large-billed birds have been observed, in winter, to feed on dormant insect larvae (mainly Diptera) hidden in reed stems that the birds manage to crush with their bills (Stegman 1956; Inseman 1990). This behaviour is limited to the populations inhabiting the southern part of the breeding range, where birds are found in reed beds throughout the year. By contrast, northern populations, which are insectivorous during the breeding season, migrate southward in autumn and feed largely on small seeds collected on the ground. In western Europe, the two morphs are parapatric during the breeding season (Blümel 1982) and sympatric during winter, when their distributions largely overlap (Amato *et al.* 1994). In northern Italy there is a contact zone between the large- and small-billed breeding populations: large-billed birds, belonging to the subspecies *intermedia*, breed in the extended reed beds of the Adriatic coast and in southern and eastern sections of the Po river valley; a few, small breeding populations are also found further south (Meschini & Frugis 1993). Small-billed birds, belonging to the nominate subspecies (*schoeniclus*), breed in the western part of the Po river valley and at the southern foot of the Alps (Brichetti & Cova 1976). Although some *schoeniclus* breeding populations are found a few kilometres away from the closest large-billed, *intermedia* breeding population, they are quite distinct morphologically, and apparently do not hybridize (A. Pilastro, unpublished results).

We report here a study of genetic variation within and between these two subspecies in their contact zone. In particular, we tried to determine whether a genetic boundary corresponds to the observed morphological boundary and, if so, how much the two morphs are genetically differentiated. For this purpose, we sampled and studied eight breeding populations, four of them of *Emberiza s. schoeniclus* and four of *E.s. intermedia*, across

the general boundary between their breeding ranges, using two genetic markers. We first used the partial sequences of two relatively slowly evolving genes of mitochondrial (mt) DNA (ND5 and *cyt-b*), which are commonly used for detecting genetic differentiation at the species and genus level. Second, we studied the allele distribution at four microsatellite loci, which have a very high mutation rate and are particularly useful for describing recent evolutionary events (Paetkau & Strobeck 1994; Paetkau *et al.* 1995; Estoup *et al.* 1995).

Materials and methods

Morphological measurements and blood sampling

One hundred and fifty individuals were captured with mist-nets, during the breeding season or during the postnuptial moult (May–August), at eight locations (Fig. 1). For each individual, bill depth was measured with callipers (to the nearest 0.1 mm) according to Svensson (1992), and body mass was measured to the nearest 0.1 g with a Pesola spring balance. As primary remiges were often extensively abraded and because several birds were moulting, wing length was measured only in a small subsample of individuals. Morphometric data were analysed using the SPSS package.

About 150 µL of blood was collected from the brachial vein of 133 individuals, preserved in isotonic solution for

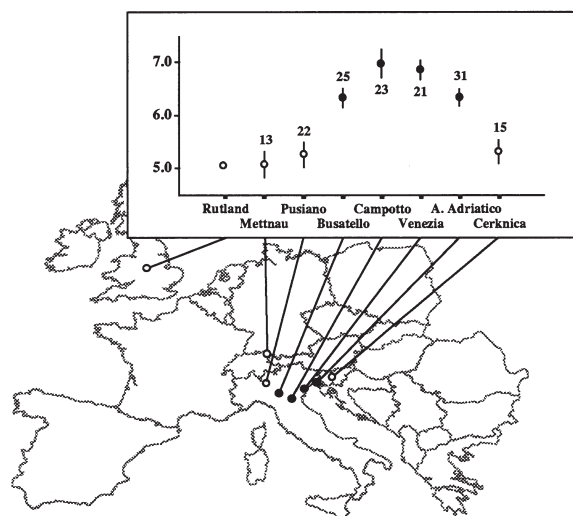


Fig. 1 Sampling location and mean bill depth (mm, \pm 99% CI) in eight reed bunting populations. For the Rutland (UK) population, for which no morphometric measurements were available, the mean bill depth for *schoeniclus* subspecies reported here is that in Cramp & Perrins (1994). All the *schoeniclus* populations had significantly smaller bills than *intermedia* ones, also after accounting for body size differences (see text). The numbers on plots indicate the size of the samples.

up to 5 days and then stored at -80°C until DNA extraction. Blood samples of 10 breeding individuals from Rutland (UK) were obtained from T. Burke, but no morphometric measurements were available for these birds. Total DNA samples were also obtained from an *E.s. schoeniclus* individual from Poland, an *E.s. pyrrhuloides* from China and from a little bunting (*E. pusilla*) and were used as outgroups. Samples were provided by Jaime Garcia Moreno of the Population Biology Department, University of Copenhagen (Denmark), and were used for the mtDNA sequence analyses.

Genetic analysis

Total genomic DNA was extracted according to the procedure of Kocher *et al.* (1989). A combination of three primers [NADW5 (5'-CACYTAATCGACCTCTCMTG-3'; C. Gilmour, ROM, unpublished), H15149 and L14990 (Kocher *et al.* 1989)] were used to amplify, using polymerase chain reaction (PCR) (Saiki *et al.* 1988), a segment of about 600 bp of the fifth subunit of NADH dehydrogenase (ND5) and cytochrome *b* (*cyt-b*). PCR was carried out in a total volume of 25 μL , containing 10 mM Tris-HCl (pH = 8.0), 1.5 mM MgCl_2 , 5 pmol each primer, 200 μM each dNTP, 0.5 units *Taq* polymerase (Perkin-Elmer Cetus) and 20–50 ng of DNA. The fragment was sequenced using the Perkin-Elmer Cetus sequencing kit, and the primers NADW5 and H15149 were labelled with [$\gamma^{33}\text{P}$]-ATP. The sequences reported here have been deposited in GenBank under Accession nos AF053413–AF053431 and AF053699.

We amplified and analysed four microsatellites: Escu1, Escu3, Escu4 and Escu6 (Hannotte *et al.* 1994), for eight populations of reed buntings; four belonging to *E.s. schoeniclus* and four to *E.s. intermedia*. All microsatellite loci used were repeats of a dinucleotide motif. The amplification solution was 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl_2 , 5% formamide, 10 pmol primer, 200 μM dNTPs, 1 unit *Taq* polymerase (Promega) and 20 ng of total DNA. Twenty-five microlitre reaction volumes were used. The amplification profile consisted of 30 cycles of: 1 min at 94°C , 1 min at 52°C (Escu3 and Escu6) or 1 min at 55°C (Escu1 and Escu4), and 1 min at 72°C . The amplification products were electrophoresed on an 8% vertical denaturing acrylamide gel. After the electrophoretic run the alleles were stained with silver nitrate (modified from Merrill 1971). Gels were then washed for 10 min in a 40% methanol and 10% ethanol solution to eliminate urea.

To determine the absolute dimensions of the alleles and facilitate comparison of gels, several samples for every locus were amplified in the presence of 10% primer R, labelled with [$\gamma^{33}\text{P}$]-ATP. These samples were run in a 6% denaturing acrylamide gel, in parallel with the sequence of a marker ($\lambda\text{gt}11$) provided with a sequencing kit (Perkin-Elmer Cetus).

Statistical analyses

cyt-b and ND5 sequences. Eleven individuals belonging to the *schoeniclus* subspecies were sequenced (four from the UK, two from Slovenia, two from Italy, two from Germany and one from Poland) and seven belonging to the *intermedia* subspecies (two from Busatello, two from Alto Adriatico, two from Venezia and one from Campotto). The sequences were aligned manually using the ESEE package (Cabot & Beckenbach 1989). The pairwise genetic distances between mtDNA sequences were obtained using the Kimura 2-parameter model (Kimura 1980), in the MEGA package (Kumar *et al.* 1993).

Microsatellite loci. The program BIOSYS (version 1.7) (Swofford & Selander 1989) was used to determine the allele frequencies in the two subspecies of reed bunting for the four microsatellite loci and also to determine the expected (Nei 1978) and observed heterozygosity for each population. The GENEPOP (version 1.2) (Raymond & Rousset 1985) package was used to compare the allele frequency distribution for the four loci between the two subspecies, to determine if the loci were in linkage disequilibrium and if the populations were at the Hardy–Weinberg equilibrium. The degree of differentiation between the two subspecies was estimated using F_{ST} , in accordance with Weir & Cockerham (1984), and R_{ST} (Slatkin 1995), as implemented in RST CALC (Goodman 1997). R_{ST} , as calculated in Slatkin (1995), assumes populations of equal sample size and loci with equal variances. This may produce biased results. For this reason, the data set was globally standardized (Goodman 1997) before carrying out the R_{ST} calculation. In this way, alleles were expressed in terms of standard deviations from the global mean rather than repeat unit number. Statistical significance for F_{ST} and R_{ST} values was estimated using Fisher's method and permutations test, respectively (Goodman 1997). Additionally, genetic differentiation between populations and subspecies was determined using the genetic distance (Nei 1978) calculated over the four loci. From the matrix of genetic distances we obtained a dendrogram using the UPGMA method (Sneath & Sokal 1973) to show graphically the relationship between populations of reed bunting. As large-billed populations sampled in this study were geographically clustered, any correlation between morphology and genetic distances may indeed be a result of gene flow between close localities and reflect geography more than morphological differentiation. We used the multiple correlation extension of the Mantel test of matrix correspondence (Smouse *et al.* 1986) to determine whether morphological and genetic distances were significantly correlated, when subject to geographical control. Essentially, this test estimates how much of the genetic pattern of differentiation is explained by morphological

differences between populations, independently of geographical distance. The significance of the coefficients was evaluated by 100 000 random permutations.

Results

Bill size

Large variation in bill depth was observed in the reed bunting populations studied. Considering the seven populations for which morphometric data were available, mean bill depth ranged between 5.08 mm (SD = 0.29, $n = 13$, Mettnau, Germany) and 7.00 mm (SD = 0.46, $n = 23$, Campotto, Italy). The difference between the smallest and the largest bill depth was 3.1 mm (4.7–7.8 mm). The birds from the three *schoeniclus* populations for which morphometric data were available (Mettnau, Germany; Pusiano, Italy; and Cerknica, Slovenia) had a smaller bill depth than the birds from four *intermedia* populations studied (5.24 mm \pm 0.35, $n = 50$; 6.61 mm \pm 0.43, $n = 100$, respectively) and the difference was highly significant (Student's t -test = 20.2, $P < 0.001$). Considering the seven populations, bill depth did not vary significantly among *schoeniclus* populations (Pusiano, Mettnau and Cerknica) whereas, among Italian *intermedia* populations, birds from Campotto and Venezia had significantly deeper bills than those captured at Busatello and Alto Adriatico (one-way ANOVA, $F = 95.5$, $P < 0.001$, least significant difference, posthoc comparisons, $P < 0.05$, Fig. 1). Birds from *intermedia* populations were, on average, significantly heavier than those from *schoeniclus* populations (mean body mass of *schoeniclus* = 18.06 \pm 1.82, $n = 48$; mean body mass of *intermedia* = 20.10 \pm 1.75, $n = 95$, Student's t -test = 6.49, $P < 0.001$). We therefore corrected for the larger body size of *intermedia* birds, using bill depth³/body mass. After correction, birds from *intermedia* still had deeper bills, on average, than those from *schoeniclus* populations (Student's t -test = 15.3, $P < 0.001$), confirming that the former have

allometrically larger bills. Considering the seven populations, new results confirmed those obtained with the rough bill depth data, i.e. all four *intermedia* populations had deeper bills than the three *schoeniclus* populations and, within *intermedia*, birds from Venezia and Campotto showed a significantly deeper bill than those from Busatello and Alto Adriatico (one-way ANOVA, $F = 70.4$, $P < 0.001$, least significant difference, posthoc comparisons, $P < 0.05$).

Genetic variability in the two subspecies of reed bunting

cyt-b and *ND5* sequences. Sequences were obtained of 515 bp (387 bp, or 33.8% of the *cyt-b*; 120 bp, or 6.6% of subunit 5 of the *ND5* dehydrogenase; and eight intergenic bases) from 19 individuals. Fifteen sites (3%) were variable (Table 1). The most common haplotype (A) was present in 13 individuals (70%) of both subspecies. Six unique haplotypes were found: three from *schoeniclus*, two from *intermedia* and one from *pyrrhuloides*. Intraspecific variation among European birds was low. The most divergent haplotype among *schoeniclus* birds (*schoeniclus* from Poland, haplotype D) differed from haplotypes of the other European reed buntings by 0.6% to 1.02% (Kimura 2-parameter model), with five substitutions occurring with respect to the most common haplotype. All the other haplotypes differed by only one or, in one case, two substitutions from the most common haplotype, and a clear pattern of genetic differentiation was not evident between large- and small-billed birds (Fig. 2). The sequence of *pyrrhuloides* differentiated more than those of the other reed buntings (14 substitutions) with an average nucleotide divergence of 2.5% (range 1.2–3.1%). Similar genetic distances between haplotypes occurred when *cyt-b* and *ND5* sequences were analysed separately.

Microsatellites. All four loci examined (Escu1, 3, 4 and 6) were highly polymorphic, with 13, 15, 19 and 18 alleles, respectively, and were polymorphic in all population

Table 1 Mitochondrial DNA haplotypes detected in the cytochrome *b* and *ND5* sequences of reed bunting. Subspecies are *schoeniclus* (Sch), *intermedia* (Int) and *pyrrhuloides* (Pyr)

Haplotype	Position no.															Subspecies		
	20	33	43	78	93	102	194	260	299	419	428	455	476	480	494	Sch	Int	Pyr
A	C	C	C	G	C	C	C	C	G	C	C	T	C	A	G	8	5	
B		T														1		
C	?	?	T	?										?		1		
D	?	?		A			T						T	G	A	1		
E							A										1	
F		?	?	A	?	?	A							?			1	
P	T	T	T	A	T	T		T	A	A	T	A	T	G	A			1

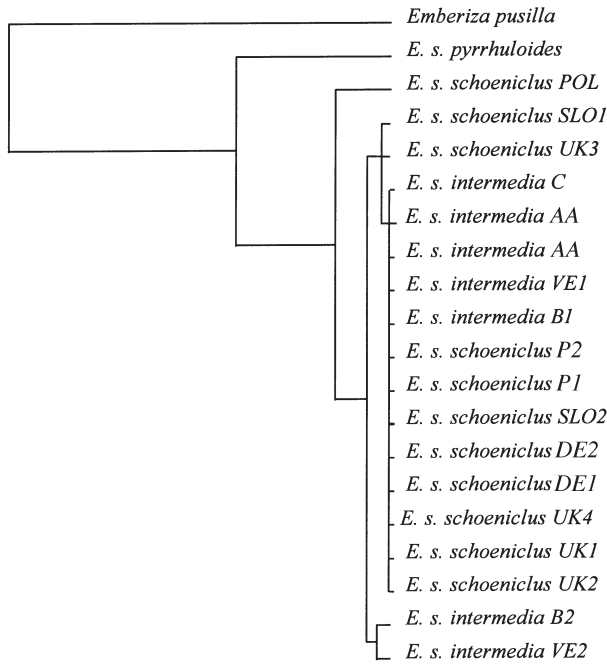


Fig. 2 Representation of the relationship between the specimens belong to reed bunting subspecies. The dendrogram was obtained with the UPGMA method based on the sequences of *cyt-b* and ND5. C = Campotto, B = Busatello, VE = Venezia, AA = Alto Adriatico, P = Pusiano.

samples. Sample size, mean number of alleles per population, mean expected and observed heterozygosity (Nei 1978) for populations are shown in Table 2. Observed heterozygosity was high and similar in all four loci (0.722–0.790). The four loci were not in linkage disequilibrium ($P > 0.05$). A significant departure from the Hardy–Weinberg equilibrium ($P < 0.001$), owing to a deficiency of heterozygotes, was observed only for locus *Escu3* in the Busatello population. Figure 3 shows the

allele frequency in the two subspecies. Distribution of alleles differed significantly between the two groups in all four loci (*Escu3*, $P < 0.05$; *Escu1*, 4 and 6, $P < 0.001$, Fisher’s exact test). F_{ST} between subspecies (0.044) was slightly larger than that observed among populations within the two subspecies ($F_{ST} = 0.028$ for *intermedia* and $F_{ST} = 0.036$ for the *schoeniclus* population, Table 3). According to these results, the number of migrants per generation (Nm), estimated using F_{ST} values, was 5.4 between subspecies, and slightly higher both within *schoeniclus* (6.7) and within *intermedia* (12.2). Using the rare alleles method (Slatkin 1985), Nm values obtained were 3.4 between subspecies, 1.7 within *schoeniclus* and 3.8 within *intermedia*. R_{ST} values (Table 3) were significantly different from zero between subspecies ($R_{ST} = 0.038$, $P < 0.001$, permutation test) and among *schoeniclus* populations but not among *intermedia* populations (respectively $R_{ST} = 0.072$, $P < 0.001$ and $R_{ST} = 0.023$, $P > 0.05$, permutation test). The number of migrants calculated using R_{ST} values were 6.3 between subspecies, 3.2 within *schoeniclus* and 10.4 within *intermedia*.

Nei’s (1978) genetic distances for the eight populations are summarized in Table 4. Mean genetic distance between subspecies, calculated on the microsatellite allele frequencies, was more than twice as high (0.48 ± 0.13 SD) as within subspecies (0.20 ± 0.08 SD). The dendrogram constructed using these distances showed two clusters corresponding to the two morphological groups (Fig. 4).

The sampled populations of *intermedia* were, on average, geographically closer to each other than those of *schoeniclus* (Fig. 1). To test whether the observed association between morphologically similar populations and the microsatellite alleles distribution was influenced by the geographically unbalanced distribution of the sampled populations of the two subspecies, a Mantel test of matrix correspondence (Smouse *et al.* 1986) was carried out on morphological, genetic and geographical dis-

Table 2 Mean number of alleles and mean expected (Nei 1978) (H_E) and observed (H_O) heterozygosity at four microsatellite loci in the eight reed bunting populations studied. The SE are shown within parentheses

Population	<i>E.s. schoeniclus</i>				<i>E.s. intermedia</i>			
	Rutland	Mettnau	Cerknica	Pusiano	Venezia	Alto Adriatico	Busatello	Campotto
Sample size	10	13	12	20	16	22	23	27
Mean no. of alleles	8.50 (0.29)	8.75 (0.48)	7.25 (0.48)	9.00 (0.71)	10.00 (1.15)	9.50 (1.19)	10.75 (0.85)	9.25 (0.85)
Average H_E	0.824 (0.030)	0.845 (0.020)	0.856 (0.012)	0.817 (0.045)	0.840 (0.049)	0.830 (0.028)	0.837 (0.022)	0.786 (0.035)
Average H_O	0.725 (0.048)	0.788 (0.066)	0.785 (0.071)	0.745 (0.074)	0.725 (0.102)	0.790 (0.031)	0.772 (0.102)	0.722 (0.081)

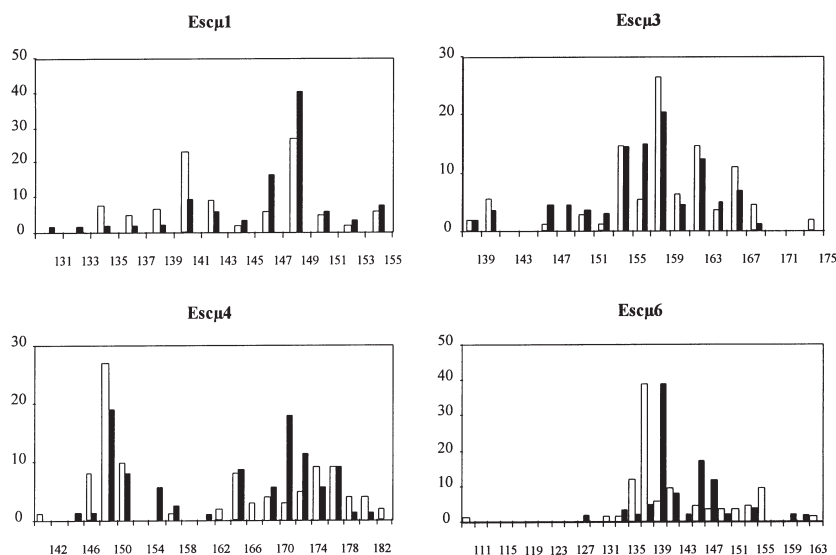


Fig. 3 Frequency distribution of alleles in two subspecies of reed bunting at four microsatellite loci. Four populations for each subspecies were pooled. Black bars represent *intermedia* subspecies and white bars represent *schoeniclus* subspecies.

tances. A significant correlation was found between geographical and genetic distance ($r=0.45$, $P=0.001$), and between genetic and morphological distance ($r=0.62$, $P<0.001$). About 20% of the total variance in the genetic distance between populations was determined by the geographical distance and 38.6% by the morphological distance, the unexplained variance being equal to 41.1%. No significant correlation was found between morphological and geographical distance matrices ($r = -0.004$, $P = 0.48$). Once the effect of the geographical distance was statistically removed using the partial Mantel test of matrix correspondence, the correlation between genes and morphology was slightly more pronounced (partial $r = 0.70$, $P < 0.001$), thus excluding the possibility that the observed association between morphology and genetics was an artefact as a result of population sampling.

Discussion

Large and highly significant variation in bill size was observed between several reed bunting populations. The population with the largest bill (Campotto, Italy) had a mean bill depth which was 38% larger than that of the population with the smallest average bill depth (Mettnau, Germany). On an individual basis, the bird with the largest bill had a bill depth which was nearly 70% larger than that of the individual with the smallest one. The geographical pattern of variation of bill size suggests a clear boundary between the two reed bunting subspecies (Fig. 1). Within the Po river valley, in northern Italy, when only bill size is considered, the breeding populations apparently belong either to *schoeniclus* (at the base of the Alps and western part of the Po valley) or to *intermedia*

Locus	Between subspecies	Among <i>schoeniclus</i> populations	Among <i>intermedia</i> populations
F_{ST}			
Escμ1	0.0225*	0.0533	0.0071
Escμ3	0.0018	0.0103	0.0521**
Escμ4	0.0167*	0.0236*	0.0158*
Escμ6	0.1315**	0.0586*	0.0335*
All loci combined	0.0444**	0.0361**	0.0277**
R_{ST}			
Escμ1	0.1155*	0.2575**	-0.0138
Escμ3	0.0329*	-0.0119	0.0102
Escμ4	0.0042	0.0053	0.0344
Escμ6	-0.0029	0.0252	0.0662*
All loci combined	0.0378**	0.0724**	0.0234

* $P < 0.05$, ** $P < 0.001$.

Table 3 F_{ST} and R_{ST} values observed at four microsatellite loci between and within two subspecies of reed bunting. The significance was obtained using Fisher's method (F_{ST}) and permutations test (R_{ST} , see Methods)

Table 4 Genetic distance (Nei 1978) (above diagonal) and SE (below diagonal) between populations in two reed bunting subspecies calculated from the allele frequencies at four microsatellite loci

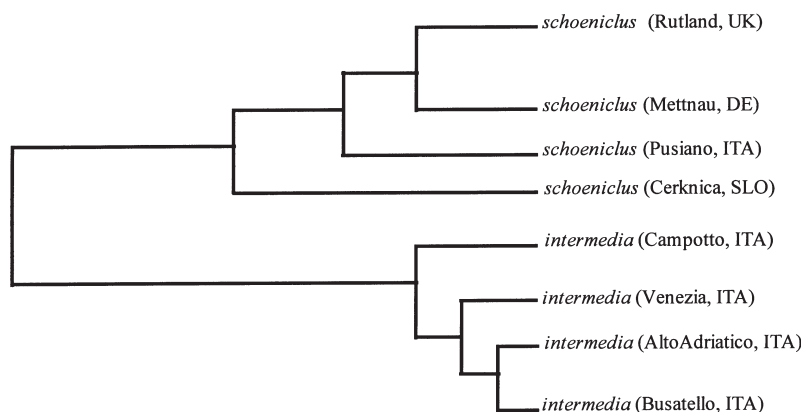
	<i>schoeniclus</i>					<i>intermedia</i>		
	Rutland	Mettnau	Cerknica	Pusiano	Venezia	Alto Adriatico	Busatello	Campotto
<i>schoeniclus</i>								
Rutland		0.1315	0.3532	0.1547	0.7243	0.5873	0.6294	0.4982
Mettnau	0.0923		0.1375	0.2249	0.6899	0.5470	0.4842	0.3551
Cerknica	0.0592	0.0998		0.3514	0.6342	0.4030	0.4308	0.3273
Pusiano	0.1117	0.1553	0.1735		0.4645	0.3523	0.3611	0.3827
<i>intermedia</i>								
Venezia	0.3847	0.2164	0.1399	0.3441		0.1359	0.1249	0.1868
Alto Adriatico	0.2659	0.2161	0.1461	0.2908	0.0965		0.1529	0.1245
Busatello	0.4295	0.2364	0.2147	0.2443	0.0273	0.0248		0.2645
Campotto	0.3411	0.1937	0.1492	0.3270	0.0869	0.0605	0.1905	

(central and eastern part of the Po valley) and, presently, no intermediate bill-sized populations are known to exist.

This steep morphological boundary between the two groups may be maintained by selection, if the advantage of having a large bill is dependent on ecological conditions that vary locally and favour only one of the two morphs in each locality. If this is the case, one must assume that the main ecological factor determining the optimal bill size has a discontinuous distribution and that this discontinuity is coincident with the boundary between large- and small-billed breeding populations. The mechanism would be substantially the same even if the factor was clinally distributed and a large bill size became advantageous only beyond a certain threshold, while an intermediate bill size was always disadvantageous. The function of the large, strong bill is likely to be related to the habit of crushing reed stems to extract insect larvae, as described in *pyrrhuloides* (Stegmann 1956; Inseman 1990). A preliminary survey of faecal content of sympatric, wintering birds of the two northern Italian

morphs confirmed the presence of insects in the diet of *intermedia* and their complete absence in that of *schoeniclus* (G. Matessi, unpublished results). The analysis of insect content of reed stems collected in winter in two localities, one in Switzerland, where *schoeniclus* breeds, and one in northern Italy, where *intermedia* breeds, suggests that the quantity of dormant insects within reed stems changes drastically from south to north of the Alps (G. Matessi, unpublished results). The morphological differentiation between the two morphs may be maintained in the presence of some gene flow, and the two subspecies may not be strongly differentiated genetically at unlinked, neutral loci.

Alternatively, the observed morphological pattern of geographical variation may be explained as the result of secondary contact between two subspecies that have been separated for long enough to allow the appearance of a different foraging specialization and the establishment of barriers to gene flow, which hamper mixing once the subspecies re-establish contact. Thus, morphological

**Fig. 4** UPGMA dendrogram of eight reed bunting populations based on Nei's (1978) genetic distances derived from allele distribution at four polymorphic microsatellite loci.

differentiation may be maintained, even in the absence of geographically varying selective forces. The evolution of specific recognition mechanisms that prevent copulation, such as differences in song or courtship behaviour, and/or postcopulatory mechanism, such as infertility of the zygote, may prevent gene flow from occurring. This would be reflected in a clear genetic differentiation between the two subspecies. A similar pattern of variation has been recently observed, for example, in a polytypic European passerine species, the chiffchaff (*Phylloscopus collybita*) (Helbig *et al.* 1995; 1996). In this species, a clear differentiation of the song (one of the most important species recognition elements in passerines) and of colouration is accompanied in parapatric populations by an average sequence divergence in mtDNA *cyt-b* close to 5%. Nothing is known about geographical variation of song in the reed bunting and the existence of other pre-copulatory or postcopulatory barriers; only scant and anecdotal information is available on the occurrence of mixed pairs (Brichetti & Cova 1976).

It is difficult to discriminate between the two scenarios described above because the selection hypothesis does not entirely rule out the secondary contact hypothesis. In fact, the two scenarios are the extremes of a series of possible intermediate situations. The observed pattern of variation of *cyt-b* and ND5 mitochondrial genes in the 18 reed buntings sequenced in this study does not allow the genetic discrimination of the two subspecies at the mtDNA level. Among the European birds, the most divergent sequence of the mitochondrial *cyt-b* and ND5 genes (in a *schoeniclus* from Poland) showed a maximum divergence of about 1%, whereas the mean divergence between the two subspecies was less than 0.5%, a value similar to that observed within subspecies. The *pyrrhuloides* haplotype of one individual from China differed from the European forms by 2.5%, which is in the range of the within-species variation observed in several passerine species studied so far (Avisé & Nelson 1989; Avisé *et al.* 1992; Taberlet *et al.* 1992; Zink & Dittman 1993; Gill *et al.* 1993; Helbig *et al.* 1995). This amount of sequence divergence is far smaller than the average value between six species of the genus *Emberiza* (10.7%) with the same mtDNA fragment, and similar to that observed within the same species of the family (1.0%, range 0–4.3%, Grapputo 1997). The relatively poor genetic differentiation between the two reed bunting subspecies studied here may be the result of either current high gene flow between populations, or a recent differentiation between the small-billed and the large-billed subspecies. The fact that one individual of *E.s. pyrrhuloides*, a large-billed subspecies from China, was only 2.5% divergent from the other two European subspecies, may be an indication that the divergence time ranged between 0.5 and 1 Myr and was therefore not as old as that observed in other passerine species

(Helbig *et al.* 1995). The habit of crushing the reed stems for feeding on insect larvae may thus be a relatively recent innovation.

Population genetic studies on passerine birds have pointed out, in some cases, the complete lack of genetic structure, even in the presence of a clear morphological variation and in the absence of any apparent gene flow. This has been related to the recent evolution of morphological differentiation (Schnell & Selander 1981; Berlocher & Bush 1982; Barrowclough 1983; Zink 1986; 1988; 1991). It may be that the genetic markers used are slowly evolving ones. Using four microsatellite loci, genetic differences between the two subspecies were more evident than those obtained from the *cyt-b* and ND5 mitochondrial sequences. The analysis of the genetic structure of the reed bunting populations using F_{ST} (Wright 1951) suggests that the amount of genetic variation between subspecies was only slightly larger than that among populations of the same subspecies. F_{ST} between subspecies (0.044) was similar to that observed in other birds using allozymes (Barrowclough 1983), but much smaller than that observed within a tropical Emberizidae species (0.132, Loughheed & Handford 1993). Nei's (1978) genetic distances were, on average, larger between populations of different subspecies than within subspecies, and the UPGMA dendrogram constructed from these distances clustered the populations according to bill size. A weak genetic differentiation between subspecies was also suggested by the R_{ST} values (Slatkin 1995), although the larger R_{ST} value among *schoeniclus* populations indicated that the geographical distance, as shown also by the Mantel test, is as important as morphology in explaining the genetic differentiation of reed bunting populations. Nonetheless, the R_{ST} and F_{ST} values and the number of migrants between subspecies and between populations within subspecies seem to indicate that there is still some gene flow between populations. Furthermore, the number of migrants per generation within the same subspecies is not substantially different from that observed between subspecies, in particular when the rare alleles method (Slatkin 1985) is used. The observed genetic structure may be either the consequence of current gene flow between subspecies or the effect of a very recent differentiation. In any case, the evolution and the maintenance of pronounced morphological differentiation between geographically close populations of the two subspecies is probably related to a strong selection for large bills in the southern part of the reed bunting's breeding range. Such selection could be related to a high winter survival rate for the large-billed birds because they can feed on more nutritious food. The two forms may not (or scarcely) interbreed, for a mechanism of sexual selection based on different songs, or the assortative mating in spring between the two forms, may be a result of differences in

the breeding phenology. The resident, large-billed form may start to breed earlier, on average, than the small-billed form. A difference of breeding phenology has been supposed by Berthold *et al.* (1992) to be the segregation mechanism underlying the rapid evolution of a novel migration habit in the German blackcaps. Our results suggest that sufficient time has elapsed for some differentiation and genetic structuring to arise at the four microsatellite loci. On the other hand, no genetic discrimination was possible with the 'slow evolving' marker (mtDNA sequence), a strong indication that large-billed reed bunting populations probably diverged in relatively recent times.

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This work was part of Alessandro Grapputo's PhD thesis under the supervision of Guglielmo Marin and Andrea Pilastro. A. Grapputo is presently a Post Doctorate at the Royal Ontario Museum, Toronto, Canada, where he is working on population genetics and behavioural ecology in birds. A. Pilastro is Assistant Professor in the Biology Department of Padova University. He is interested in evolutionary and behavioural ecology of vertebrates, in particular, sexual selection and reproductive strategies. G. Marin is Professor of Ethology at Padova University. His interests include the use of molecular approaches in the study of the population and evolutionary biology of vertebrates.
