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Genetic and morphological variation in a Mediterranean glacial refugium: evidence from Italian pygmy shrews, *Sorex minutus* (Mammalia: Soricomorpha)

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At the Last Glacial Maximum (LGM), the southern European peninsulas were important refugia for temperate species. Current genetic subdivision of species within these peninsulas may reflect past population subdivision at the LGM, as in 'refugia within refugia', and/or at other time periods. In the present study, we assess whether pygmy shrew populations from different regions within Italy are genetically and morphologically distinct. One maternally and two paternally inherited molecular markers (cytochrome *b* and Y-chromosome introns, respectively) were analysed using several phylogenetic methods. A geometric morphometric analysis was performed on mandibles to evaluate size and shape variability between populations. Mandible shape was also explored with a functional approach that considered the mandible as a first-order lever affecting bite force. We found genetically and morphologically distinct European, Italian, and southern Italian groups. Mandible size increased with decreasing latitude and southern Italian pygmy shrews exhibited mandibles with the strongest bite force. It is not clear whether or not the southern Italian and Italian groups of pygmy shrews occupied different refugia within the Italian peninsula at the LGM. It is likely, however, that geographic isolation earlier than the LGM on islands at the site of present-day Calabria was important in generating the distinctive southern Italian group of pygmy shrews, and also the genetic groups in other small vertebrates that we review here. Calabria is an important hotspot for genetic diversity, and is worthy of conservation attention. *Biological Journal of the Linnean Society*, 2010, **100**, 774–787.

ADDITIONAL KEYWORDS: Calabria – cytochrome *b* – geometric morphometrics – refugia – Italy – phylogeography – Y-chromosome introns.

INTRODUCTION

The three southern European peninsulas, namely the Iberian, Italian, and Balkan (Fig. 1), acted as refugial areas for many species during the Quaternary ice ages, and are consequently species-rich areas and current hotspots of intra-specific diversity (Bilton *et al*., 1998; Hewitt, 2000; Myers *et al*., 2000; Petit *et al*., 2003). Traditionally, the southern peninsulas have been considered as single refugial areas at the Last Glacial Maximum (LGM; Bilton *et al*., 1998; Petit *et al*., 2003). However, recent studies indicate that species in the southern European peninsulas may have persisted in multiple distinct and separated

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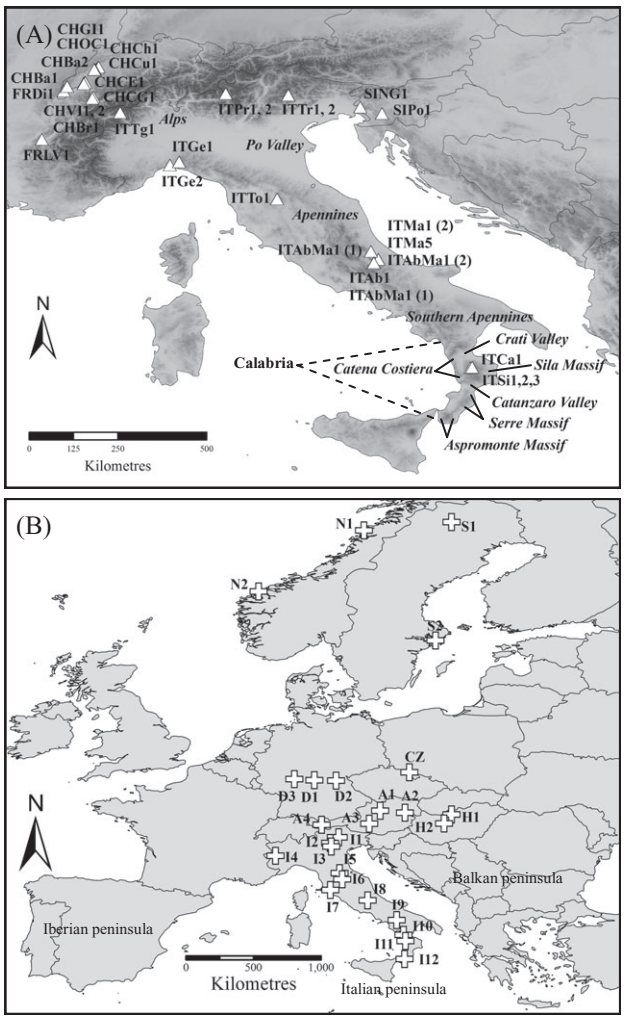

Figure 1. Sample localities for the pygmy shrew in Italy and elsewhere for (A) the cytochrome *b* data, with relief illustrated in grey tones, and (B) the morphometric data (samples from western Siberia are not mapped).

glacial refugia in Iberia (Gómez & Lunt, 2006), Italy (Canestrelli, Cimmaruta & Nascetti, 2007, 2008), and the Balkans (Kryštufek *et al*., 2007). If this pattern is common, it has important consequences for the interpretation of European phylogeography as well as our understanding of biological diversity.

In the present study, we consider genetic and morphological subdivision in one species of small mammal in the Italian refugial area and compare this with other small vertebrate species from the same area. Our focal species is the pygmy shrew, *Sorex minutus* Linnaeus 1766 (Mammalia, Soricomorpha), which has a wide Palaearctic distribution extending between north-western Spain and Lake Baikal in Siberia (Hutterer, 2005). The distribution of the species becomes patchy and limited to higher altitudes in southern Europe and taxonomists have associated this trend with differentiation. Hutterer (1990) recognized five valid subspecies: *Sorex minutus minutus* (northern and central Europe to Siberia), *Sorex minutus gymnurus* Chaworth–Musters, 1932 (Greece), *Sorex minutus becki* von Lehmann, 1963 (the Alps), *Sorex minutus carpetanus* Rey, 1971 (Spain), and *Sorex minutus lucanius* Miller, 1909 (the Basilicata and Calabria ridges of southern Italy; Fig. 1A). However, he did not resolve the taxonomic status of the pygmy shrew populations in central Italy.

Phylogeographic analyses of the pygmy shrew using mitochondrial DNA (mtDNA) and Y-chromosome introns have revealed a genetic structure over Eurasia that is considered to reflect isolation and differentiation in different refugia (Bilton *et al*., 1998; Mascheretti *et al*., 2003; McDevitt *et al*., 2010). At least one glacial refugium in Italy has been proposed to explain the genetic distinctiveness of pygmy shrews existing there compared to the rest of Eurasia, and McDevitt *et al*. (2010) described a single individual with distinct mtDNA and Y-chromosome introns from southern Italy.

Thus, studies on the pygmy shrew provide evidence of lineage diversification of Italian populations from the rest of Eurasia and likely genetic and morphological subdivision within Italy. To extend these findings, we used two approaches: First, as previously employed, we implemented a phylogeographic approach, which is useful for exploring the principles and processes that generated the geographic distribution of genealogical lineages (Avise, 2000). The phylogenetic relationships among pygmy shrew populations were determined by an analysis of maternally and paternally inherited markers, adding new samples to the pre-existing data. Additionally, we used a geometric morphometrics approach (Rohlf & Marcus, 1993) on pygmy shrew mandibles from throughout Italy and neighbouring countries. Geometric morphometrics is one of the most powerful techniques for the description and interpretation of patterns of variation below the species level (Loy, 1996; Zelditch *et al*., 2004). Because populations of pygmy shrews are scattered across a variety of different environments in Italy, adaptation may be a major driving force of recent morphological evolution in this region. Therefore, morphological variability was studied in the context of a functional hypothesis that envisages mandible shape as a first-order lever affecting bite force.

In the present study, we have also considered the genetic and morphological variation in pygmy shrews from Italy in the wider context of European biodiversity. The Italian peninsula hosts a large number of terrestrial species of mammals (74 native species), five of which are endemic, thus accentuating Italy's role and responsibility in European conservation (Gippoliti & Amori, 2002). Our phylogeographic and

morphological study, in conjunction with other data on small mammals that we review here, has important implications for the conservation of Italian mammals.

MATERIAL AND METHODS

Phylogeographic analysis

In total, 35 pygmy shrews were used for the cytochrome *b* (cyt *b*) analysis (Fig. 1A; see also Supporting Information, Table S1). Tissue samples were obtained from 16 individuals from several regions in northern, central, and southern Italy, plus 14 individuals from neighbouring parts of France, Switzerland, and Slovenia. Five published cyt *b* sequences of *S. minutus* (AJ535420–AJ535424) from Italy, France, and Switzerland and a sequence of *Sorex volnuchini* (used as an outgroup; AJ535458) were obtained from GenBank (Mascheretti *et al*., 2003). Additionally, two Y-chromosome intron sequences (DBY-7 and UTY-11; Hellborg & Ellegren, 2003) were obtained from 15 male *S. minutus* from Italy and neighbouring regions, plus five DBY-7 and UTY-11 *S. minutus* sequences and a sequence of *S. volnuchini* (used as an outgroup) from McDevitt *et al*. (2010) (see Supporting Information, Table S1).

Genomic DNA was extracted using a commercial kit (Qiagen). Partial cyt *b* sequences were obtained by polymerase chain reaction (PCR) amplification either using two primer pairs that amplified approximately 700 bp of overlapping fragments or using five primer pairs (for museum samples with highly degraded DNA) that amplified approximately 250 bp of overlapping fragments (see Supporting Information, Table S2). PCR amplification for cyt *b* was performed in a 50-μL final volume: 1× buffer, 1 μM each primer, 1 μM dNTPs, 3 mM $MgCl_2$ and 0.5 U Platinum *Taq* Polymerase (Invitrogen), with cycling conditions of 94 °C for 4 min, 40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and a final elongation step at 72 °C for 7 min. Purification of PCR products was conducted with a commercial kit (Qiagen) and sequenced (Macrogen and Cornell University Core Laboratories Center). Amplification conditions of the Y-chromosome introns are described elsewhere (Hellborg & Ellegren, 2003) and the sequences were concatenated. Known female and male samples were used as controls in all PCR reactions for Y-chromosome introns.

Sequences were edited in BIOEDIT, version 7.0.9.0 (Hall, 1999), aligned in CLUSTALX, version 2.0 (Larkin *et al*., 2007), and collapsed into haplotypes using DNASP, version 4.90.1 (Rozas *et al*., 2003). The model of evolution that best fit the molecular data was determined using MODELTEST, version 3.7 (Posada & Crandall, 1998), using the minimum Akaike's information criterion value. For cyt *b*, the supported substitution model was Hasegawa–Kishino–Yano 1985 with a proportion of invariable sites of 0.5308, a gamma correction of 0.8018, and nucleotide frequencies of $A = 0.2699$, $C = 0.2981$, $G = 0.1417$, and $T = 0.2903$. For the concatenated Y-chromosome introns, the best model was Tamura–Nei 1993 with an equal proportion of invariable sites, no gamma correction, and nucleotide frequencies of $A = 0.3313$, $C = 0.1957$, $G = 0.1901$, and $T = 0.2828$. The phylogenetic relationships among cyt *b* and Y-chromosome intron haplotypes were inferred using different methods in PAUP*, version 4.0b10 (Swofford, 2000), complemented with PAUPUp, version 1.0.3.1 (Calendini & Martin, 2005): neighbour-joining (NJ) and maximum likelihood (ML), using the appropriate evolutionary model, and maximum parsimony (MP), using a heuristic search with simple stepwise addition of taxa and branch swapping (tree bisection reconnection, one million rearrangements). Statistical support for the phylogenetic relationships was assessed by 10 000 bootstrap replicates for NJ and 1000 bootstrap replicates for MP and ML. A Bayesian analysis was performed in MrBayes, version 3.1 (Huelsenbeck & Ronquist, 2001), using the appropriate evolutionary model. Two runs were performed with one million generations, a sampling frequency every 100 generations (to give a total of 10 000 samples for each run), a temperature of 0.1 for the heated chain, and checked for convergence. Trees were summarized after a burn-in value of 2500 to obtain the posterior probabilities of each phylogenetic split. A phylogenetic network was constructed using NETWORK, version 4.5.1.0 (Fluxus-Engineering), with a median-joining algorithm and a greedy genetic distance calculation method.

The cyt *b* haplotypes that clustered into major genetic lineages with high bootstrap support (or high posterior probabilities) were considered as distinct phylogroups (Avise, 2000). Genetic diversity values (π, nucleotide diversity of Jukes–Cantor) for each phylogroup and for the total sample were calculated using DNASP. Genetic divergence values between all pairs of phylogroups were estimated as *Da* (the mean ± SD number of net nucleotide substitutions per site between phylogroups with Jukes–Cantor correction) using DNASP. Divergence times between cyt *b* phylogroups were estimated as $T = Da/2\mu$, where 2μ is the divergence rate (again given along with the SD). We used the divergence rate of 2% per Myr assuming equal rates of mtDNA sequence divergence among phylogroups (Taberlet *et al*., 1998). ARLEQUIN, version 3.11 (Excoffier, Laval & Schneider, 2005), was used to estimate the pairwise genetic differentiation values (F_{ST}) between all pairs of phylogroups, for an analysis of molecular variance (AMOVA) among and

within phylogroups, and for a locus-by-locus (per nucleotide site) AMOVA. Ten thousand nonparametric permutations were performed to generate a random distribution to test the significance of the pairwise F_{ST} values and covariance components of the AMOVA, and $\alpha = 0.01$ was set as the threshold for statistical significance. No estimates of genetic diversity or differentiation were made for the Y-chromosome introns because of the low number of males per clade.

Geometric morphometric analysis

We examined a total of 277 mandibles of pygmy shrews from 99 different localities in Europe and western Siberia. Because most of the localities were represented by a small number of specimens, samples were pooled on the basis of geographic proximity resulting in 27 operational taxonomic units (OTUs) (Fig. 1B; see also Supporting Information, Table S3). Images of mandibles were collected using a Pixera Professional camera (Pixera Corporation) at a 1.2 million pixel resolution equipped with a Nikkor 210 mm APO MACRO lens, at a fixed distance of 93 cm. Morphological analyses were carried out using the 'tps-Series' software (developed by F.J. Rohlf, Department of Ecology and Evolution, State University of New York at Stony Brook, NY, USA; all software are available at: http://life.bio.sunysb.edu/morph/).

Fourteen landmarks were digitized from the internal side of each mandible (Fig. 2A) using tpsDig. The size of each mandible was estimated using the centroid size (CS) (i.e. the square root of the sum of squared distances between each landmark and the centroid) (Bookstein, 1991), obtained by the software tpsRelw and was natural log-transformed. The landmark configurations were scaled, translated, and rotated using generalized Procrustes analysis (GPA; Rohlf & Slice, 1990). A weight matrix (W) incorporating uniform ($N = 2$) and non-uniform ($N = 22$) components was extracted using GPA (Bookstein, 1996). Both components were interpreted as shape variables ($N = 24$) and then reduced through a principal component analysis [namely, relative warp (RW) analysis] using tpsRelw.

Because of the relatively small number of individuals in each OTU, we first tested for size and shape differences between the sexes to justify pooling of sexes in subsequent analyses. We analyzed the effect of sex, OTUs, and their mutual interaction in the OTUs CZ and I9, which had an adequate number of males ($N = 13$ and $N = 12$, respectively) and females ($N = 7$ and $N = 15$, respectively). The effect of sexual dimorphism was examined by analysis of variance (ANOVA) on CS (for size) and by multivariate analysis of variance (MANOVA) on W (for shape).

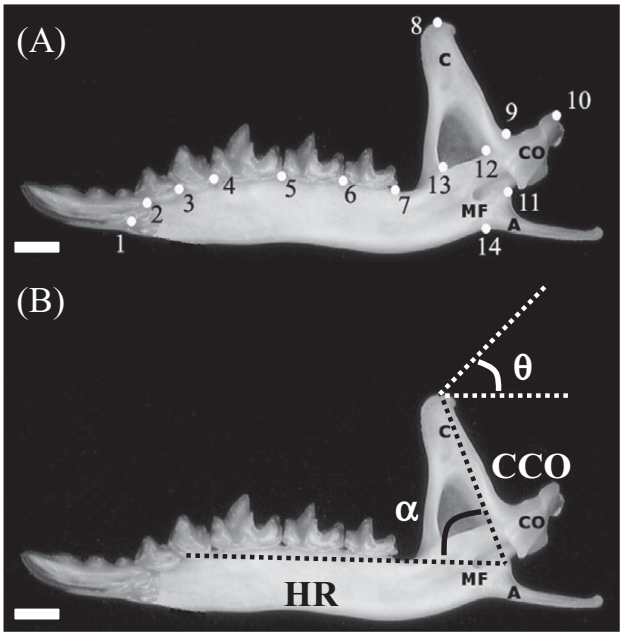

Figure 2. Location of 14 landmarks placed on the internal side of mandibles of the pygmy shrew (A) and measurements for bite force as a first-order lever on pygmy shrew mandibles (B), where coronoid–condyloid length (CCO) is the in-lever and horizontal ramus length (HR) is the out-lever, α is the angle between CCO and HR, and $\theta = 90° - \alpha$. The bite force is measured as $(\cos\theta \times CCO)/HR$. MF, mandibular fossa; C, coronoid process; CO, condyloid process; A, angular process; white bar = 1 mm.

The pattern of shape variation related to mandible size change (allometry) was compared for the full data set using tpsRegr. A correlation was performed to assess how OTUs mean CS varied with latitude (in decimals). The size differences among OTUs and among phylogroups (dividing the full mandible data set into phylogroups detected by the phylogeographic approach) were evaluated by ANOVAs and visualized with box plots. Levene's tests were performed to detect heteroscedasticity; however, the ANOVA is sufficiently robust to the violation of the assumption of homogeneity of variances (Zelditch *et al.*, 2004). We performed a Tukey–Kramer post-hoc test because it allows for unequal sample size (Sokal & Rohlf, 1995). Shape differences among OTUs and phylogroups were evaluated via MANOVAs on W, followed by a Hotelling T^2 test for multivariate comparisons.

Ordination of the OTUs was obtained through RW analysis (Rohlf, 1993) on the consensus (average) configurations of the 27 OTUs using the software tpsRelw. Shape changes in the RW space were visualized as thin-plate spline deformation grids (Bookstein, 1989). Procrustes distances among the consensus configurations of the OTUs were then

computed using tpsSmall and entered into NTSYS, version 2.2 (Exeter Software), to produce a dendrogram using an unweighted pair group method with arithmetic mean (UPGMA) to evaluate the phenetic relationships.

A functional adaptive hypothesis for shape variation in the mandible (represented as thin-plate spline deformation grids) was investigated by estimating the bite force (BF) in the context of a first-order lever (Fig. 2B; Fearnhead, Shute & Bellairs, 1955; MacDonald & Burns, 1975; Carraway & Verts, 1994), where the in-lever is the coronoid–condyloid length (CCO) measured from the tip of the coronoid process (C; landmark 8) to the base of the condyloid process (CO; landmark 11), and where the out-lever is the horizontal ramus length (HR) measured from CO to the facet-tip of M_1 (landmark 3; Fig. 2B). A stronger bite is given by the increased ratio between the in-lever (CCO) and the out-lever (HR), or by altering the angle (α) between them such that it becomes more obtuse, as described by $BF = (\cos\theta \times CCO)/HR$, where $\theta = 90° - \alpha$. CCO, HR, and α were measured on the deformation grids for the extreme of shape variation along the first RW (RW1) (i.e. the one that explained most of the variation).

RESULTS

Phylogeographic analysis

We analysed a cyt *b* fragment of 1110 bp for the 35 pygmy shrews. There were 31 haplotypes (27 haplotypes first reported in the present study; GenBank accession numbers: GQ272492–GQ272518) with 103 polymorphic sites (105 mutations and one complex codon, with three mutations not included in the analysis) of which 48 were parsimony informative. In total, there were 92 synonymous and ten nonsynonymous changes.

For the Y-chromosome introns, we analysed a concatenated fragment of 1143 bp. We found only five haplotypes that together had 11 polymorphic sites (of which nine were parsimony informative) and one site with an insertion/deletion (GenBank accession numbers: GQ272519–GQ272521 for DBY-7 and GQ272522–GQ272526 for UTY-11). Two of the haplotypes have been reported previously (see Supporting Information, Table S1).

The different phylogenetic methods produced topologically similar trees for the cyt *b* data with high bootstrap and posterior probability support for particular branches (Fig. 3A), classified as: (1) a 'European' phylogroup including samples from Switzerland and strictly northern regions of Italy in the Alps (Sondrio, Piemonte and Trento); (2) an 'Italian' phylogroup (northern–central Italy) including samples from central regions (Abruzzo) and north-western regions (Genova) in the Apennines, but also including samples from the north and north-east of Italy (Alps), Switzerland, and neighbouring regions in France and Slovenia; and (3) a 'southern Italian' phylogroup with samples strictly from Calabria (La Sila mountain). Of all the phylogroups, the southern Italian had the greatest bootstrap and posterior probability support with the various phylogenetic methods. The sequences from the whole Italian peninsula (including both the 'Italian' and 'southern Italian' phylogroups) also formed a well supported clade. One sample from Switzerland (CHCE1) clustered separately from all the rest and has been identified as belonging to a distinct Balkans lineage (R. Vega & J. B. Searle, unpublished data), and is not considered further here. The same phylogroups were also seen in the phylogenetic network (data not shown), with the European phylogroup separated from the Italian and southern Italian phylogroups by 18 mutational steps and the southern Italian and Italian phylogroups separated by 12 steps.

Similarly, the phylogenetic analysis of concatenated Y-chromosome introns (Fig. 3B) revealed a distinct 'southern Italian' lineage (one haplotype, four samples). This was most closely related to an 'Italian' lineage (one haplotype, six samples) composed of northern–central Italian and Swiss samples that in the cyt *b* trees also formed an Italian phylogroup. Additionally, there was a 'European' lineage composed of eight Swiss samples, six of which clustered within the cyt *b* European phylogroup, CHVI2 that clustered within the cyt *b* Italian phylogroup and CHCE1 that belonged to a cyt *b* Balkans lineage, plus ITPr2 (Prasota, northern Italy) that clustered within the European cyt *b* phylogroup and SIPo1 (Postjoma, Slovenia) that clustered within the Italian cyt *b* phylogroup.

The nucleotide diversity (π) was 0.0157 for the cyt *b* data overall. For the European phylogroup, $\pi = 0.0057$ relating to 11 haplotypes (all nonredundant sequences). For the Italian phylogroup, $\pi = 0.0063$ and there were 15 haplotypes, two occurring in multiple individuals (samples from Abruzzo). For the southern Italian phylogroup, $\pi = 0.0073$, reflecting four different haplotypes, although all samples were caught in the same area (La Sila mountain) and had 14 mutations that were not shared with other phylogroups (six of which were significantly differentiated from the rest of the sample by a locus-by-locus AMOVA and sequence comparison). For the whole Italian phylogroup (Italian plus southern Italian), $\pi = 0.0096$ relating to 19 haplotypes.

The divergence between phylogroups was small, as expected for within species comparisons. For European versus Italian $Da = 1.6\%$ ($\pm 0.3\%$), comparable to

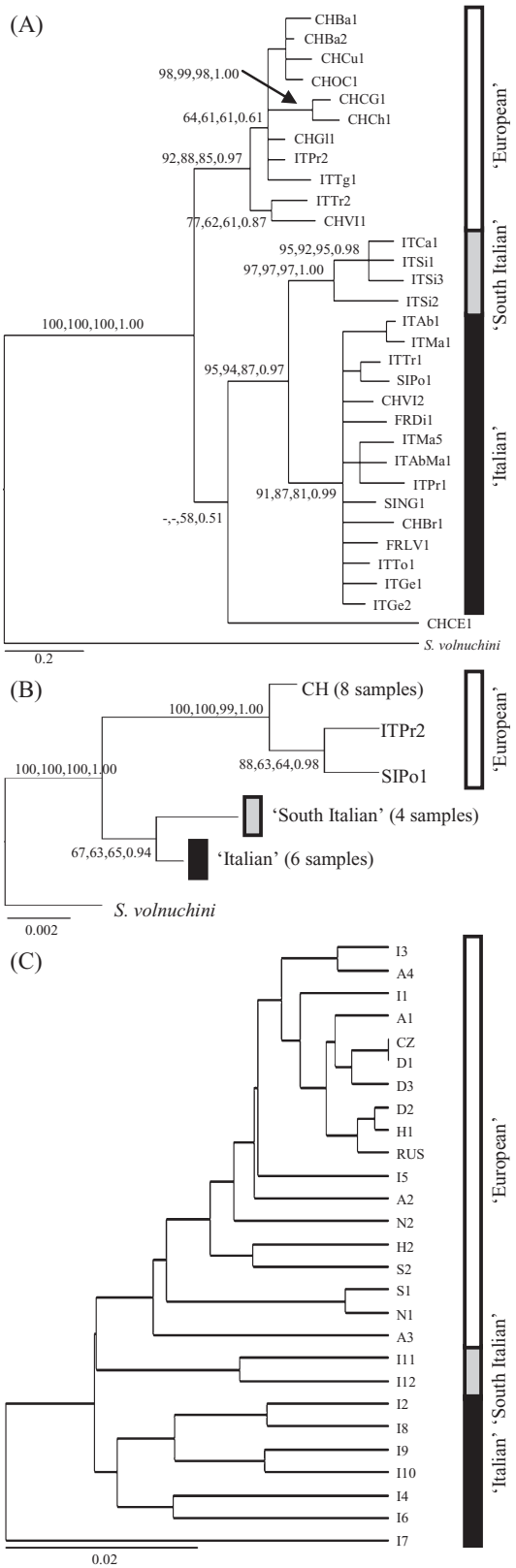

Figure 3. Bayesian analysis of cytochrome *b* data (A) and concatenated Y-chromosome introns DBY-7 and UTY-11 (B) for pygmy shrews with distinct lineages highlighted. C, unweighted pair group method with arithmetic mean phenogram of Proscrustes distances among the consensus configurations of 27 operational taxonomic units showing shape differences between 'European', 'Italian' and 'southern Italian' pygmy shrew mandibles. Values on phylogenetic trees correspond to branch support for neighbour-joining, maximum parsimony maximum likelihood and Bayesian analysis, respectively.

European versus southern Italian $Da = 1.5\%$ (±0.4%), whereas Italian versus southern Italian had the smallest divergence, $Da = 1.0\%$ (±0.3%). For the European versus whole Italian sample (Italian plus southern Italian) $Da = 1.4\%$ (±0.2%). All divergence times among phylogroups pre-dated the LGM according to the divergence rate used, where $T_{(\text{European-Italian})} = 0.8 \pm 0.15$ Mya, $T_{(\text{European-southern Italian})} = 0.75 \pm 0.2$ Mya, $T_{(\text{Italian-southern Italian})} = 0.5 \pm 0.15$ Mya, and $T_{(\text{European-whole Italian})} = 0.7 \pm 0.3$ Mya. The AMOVA showed that 71% of the overall genetic variation could be attributed to differences among the three phylogroups and 29% to within-phylogroup variation ($F_{ST} = 0.7101$, $P < 0.001$). Pairwise F_{ST} values were high and significant between phylogroups as a result of the 27 significantly differentiated polymorphic nucleotide sites among populations as determined by the locus-by-locus AMOVA and sequence comparison.

Geometric morphometric analysis

The MANOVA on the *W* matrix revealed significant mandible shape differences between the OTUs CZ and I9 but no significant effect of sex and the interaction sex × OTU. Therefore, all subsequent analyses on shape were performed pooling all samples irrespective of sex.

The regression of shape on size (allometry) was significant ($F_{24, 6600} = 3.03$; $P < 0.001$). The ANOVA on CS among OTUs was significant ($F_{26, 273} = 12.647$, $P < 0.001$); however, Levene's test was significant and so this result has to be taken with caution and no post-hoc comparisons among OTUs were made. The ANOVA on CS among the mandible sample divided into phylogroups was significant ($F_{2, 271} = 53.212$, $P < 0.001$), and homogeneity of variances supported. Post-hoc tests showed significant CS differences among all pairs of phylogroups, although caution is warranted because of the limited number of mandibles available for the southern Italian phylogroup. There was a significant inverse correlation between mandible size and latitude ($r = -0.661$; $P < 0.001$).

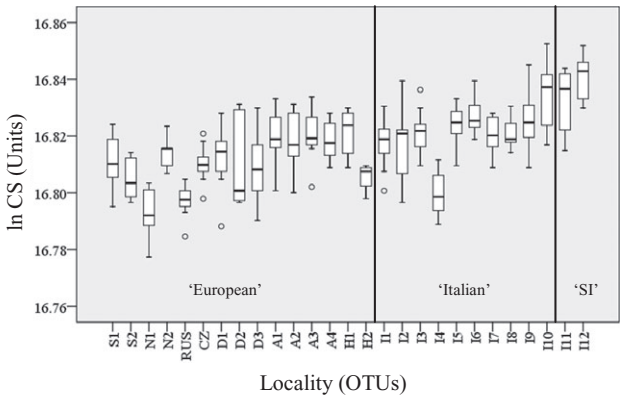

Figure 4. Box-plot showing mandible centroid size (CS) variation (natural log-transformed) of 27 operational taxonomic units of pygmy shrew ordered according to latitude from north (left) to south (right), and by phylogroups (SI, southern Italy).

Mandible size increased progressively from northern to southern European localities (Fig. 4). The only obvious outlier was the north-western Italian locality I4 (Torino), which fell within the range of northern–central European populations.

The MANOVA on *W* among OTUs were significant (Wilk's $\lambda = 0.008$, $P < 0.001$). The Hotelling T^2 test showed that 51% of the between group comparisons were significant. The highest percentage (80–100%) of significant differences involved comparisons of central–southern Italian OTUs both among themselves and against northern–central European OTUs. The MANOVA on *W* divided into phylogroups was significant (Wilk's $\lambda = 1.592$; $P = 0.0057$); however, Hotelling's T^2 test showed significant shape differences only between the European and Italian phylogroups ($P = 0.0027$). The southern Italian phylogroup was not different from either of these groups ($P = 0.1467$), most likely as a result of small sample size.

The first three RWs computed on the OTU consensuses explained cumulatively 61.75% of the total variance (28.90%, 20.51%, and 12.34%, respectively). There were two groups identifiable based on the geographic origin of the OTUs (Fig. 5A): a European group including all OTUs from northern and central Europe plus north-eastern Italian OTUs I1 and I3 (Trentino, Alps) and central Italian OTU I5 (Foreste Casentinesi, Apennines), and an Italian group with OTUs I2, I4, I6, and I8 to I12. OTU I7 (Grosseto, Tuscany) was an outlier. There was a higher diversity for the mandible within the Italian group along RW2 than within the European group. However, the southern Italian OTUs (I11 and I12) could not be distinguished from the central and northern Italian OTUs based solely on RW analysis.

The main shape differences between the two groups found with RW analysis (Italian and European) are shown, as an example, by the deformation grids taken from the most extreme values along the RW1 axis (the RW that explained most of the variation) from each group (Fig. 5B, C). There was a stronger BF in Italian compared to European pygmy shrews (BF = 0.238 and 0.074, respectively). The mandible of Italian (Fig. 5B) and European shrews (Fig. 5C) was characterized by different slopes of the incisive alveolus (landmark 2), a different HR length, and by notable changes in the posterior portion affecting the relative position of the masseteric fossa (MF) and the angular process (A). The greatest BF in Italian shrews was given by an apparent lengthening of C and a shorter HR length, which increased the ratio between the in-lever and out-lever compared to European pygmy shrew mandibles. However, in Italian shrews, there were relative movements of CO (landmarks 9, 10, and 11) and C in opposite directions causing a forward shift of its tip (landmark 8) and a more acute angle α (between CCO and HR) compared to European pygmy shrew mandibles.

The UPGMA phenogram showed morphological clusters that divided the samples into three main distinct geographical regions (Fig. 3C): (1) a 'European' cluster with northern–central European and north-eastern (Alpine) Italian OTUs (I1 and I3), but which also included OTU I5 (central Italy); (2) an 'Italian' cluster with central Italian OTUs and Italian OTUs from the southern Apennines (Collemeluccio, Muro Lucano and Catena Costiera); and (3) a strictly 'southern Italian' cluster with Calabrian samples (OTUs I11 and I12); OTU I7 (Grosseto, Tuscany) was an outlier.

DISCUSSION

Morphological and phylogeographical congruencies

The present study represents the first combined phylogeographic and morphological analysis of the pygmy shrew, and it had a focus on populations of the species in the Italian peninsula. In general, we found congruence between phylogeographic and morphological patterns. Both analyses consistently identified pygmy shrews from the Italian peninsula as being different from northern–central European pygmy shrews, and further distinguished southern Italian pygmy shrews of the Calabria region (Fig. 1A) as being different from Italian populations further north.

We found three main geographically coherent genetic and phenetic clusters (Fig. 3A, B, C). The European morphological group (based on geometric

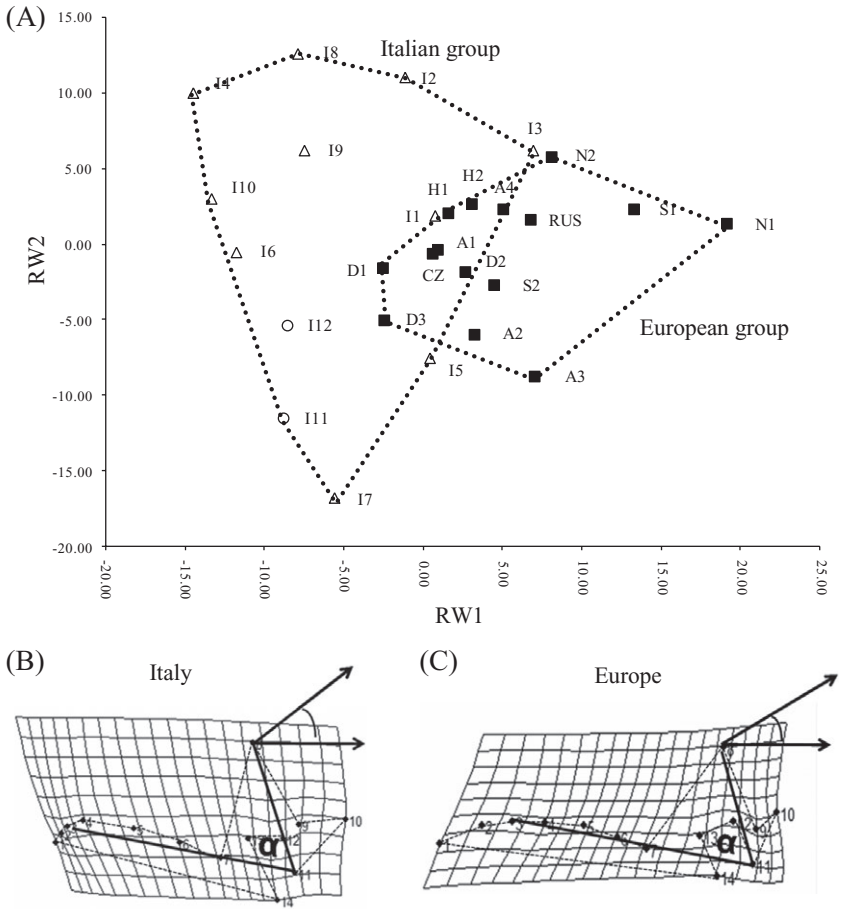

Figure 5. Morphometric analyses of the mandibles of pygmy shrews. Ordination of the consensus configurations (A) of 27 operational taxonomic units (OTUs) along the first two relative warp (RW) axes. ■, Northern–central European OTUs; Δ, northern–central Italian OTUs; ○, southern Italian OTUs. Shape changes and bite force implied by the variation along RW1 are shown as thin plate spline deformation grids representing the extremes of variation along the axis for (B) an Italian OTU and (C) a European OTU, respectively.

morphometrics) had a widespread distribution in Eurasia (from western Siberia to Norway and Hungary) and included samples from the northern regions of Italy. Previous data show that the European genetic group (based on cyt *b* and the Y-chromosome introns), which we found in central European and north Italian samples, is similarly very widespread (Bilton *et al*., 1998; McDevitt *et al*., 2010; Mascheretti *et al*., 2003). The Italian phenetic cluster included mandible samples from central regions of the peninsula together with OTUs from the Italian Alps and from the southern Apennines. Likewise, the Italian genetic group consisted of north Italian samples from the Alps and central Italian samples from the Apennines. However, more genetic sampling is desirable to determine whether this phylogroup reaches the southern Apennines. The situation in northern parts of Italy is also worth further study. Genetic and morphological samples from geographically close localities in northern Italy either belonged to the European or to the Italian group (e.g. for the genetic data, samples from Trento and Prasota; for the morphological data, OTUs I1, I2, and I3). Apparently this is a contact area of two lineages.

Interestingly, we found a genetically distinct phylogroup of pygmy shrews from the Calabria region in southern Italy for cyt *b* and Y-chromosome data (all samples from La Sila). We also found a morphologically distinct group of pygmy shrews from this region, specifically from La Sila (I11) and Aspromonte (I12) massifs. When the morphological samples were pooled into the same groupings as the genetic phylogroups, the southern Italian samples had significantly larger mandibles by centroid size than other phylogroups. The southern Italian morphological samples also clustered separately by shape but, when there was no a priori grouping of OTUs, these

samples were not significantly different from northern–central Italian samples by MANOVA and RW analysis. The southern Italian morphological samples from Collemeluccio (I8), Muro Lucano (I9), and Catena Costiera (I10) clustered with northern–central Italian samples and not with those from La Sila and Aspromonte, suggesting that the morphologically and genetically distinct population of pygmy shrews in southern Italy has a very limited distribution (Fig. 1A). More extensive sampling in this southern region (Catena Costiera, other parts of the southern Apennines in Basilicata region, and further south from La Sila in the Serre and Aspromonte massifs) is desirable to obtain a more precise morphological, phylogeographic, and population genetic description. Nevertheless, the genetic results obtained in the present study indicate strongly the distinctiveness of the southern Italian phylogroup. The phylogenetic results were consistent among methods and the phylogroups displayed high branch support and were significantly differentiated. Moreover, in the few samples from southern Italy, there were several fixed and significantly differentiated mutations that were not shared with other phylogroups, and even the less variable Y-chromosome introns revealed a distinct southern Italian lineage. Collectively, the results display the phylogeographic and morphological distinctiveness of pygmy shrew populations from the Italian peninsula and the Calabria within it.

We found some discrepancy between the morphological and genetic analyses. For the morphological data, OTU I5 from central Italy (Foreste Casentinesi) clustered within the European group, whereas, for the genetic data, no central Italian sample clustered within the European lineage. The Italian cyt *b* phylogroup contained several central European samples from Switzerland, Slovenia, and France, whereas no European morphological sample grouped within the Italian cluster. Also, MANOVAs on mandibles among OTUs mainly differentiated European from central–southern Italian samples and the RW analysis discriminated these two main groups only, whereas pairwise F_{ST} on genetic data showed significant differences among European, Italian, and southern Italian lineages.

OTU I7 was an outlier in the cluster and RW analyses and, to further understand this, we need better sampling along the Tyrrhenian coast. Discrepancies among maternally and paternally inherited markers, specifically in northern Italy and Switzerland, may be explained by the larger dispersal rate and activity areas of male versus female pygmy shrews (Shchipanov *et al.*, 2005), as expressed in the contact zone of distinct lineages, and by different mutation rates of the markers examined.

Size and bite force among morphological groups

The mandible of pygmy shrews increases in size from north to south and represents an exception to Bergmann's rule, as reported for other Soricinae in Europe (Ochocinska & Taylor, 2003), where it was suggested that small body size is adaptive under conditions of low resource availability in northern latitudes, especially in winter.

The mandible of shrews may be considered as a first-order lever during bite and mastication (MacDonald & Burns, 1975). This is because the lower condyloid facets act as the fulcrum with the in-lever (CCO) set at an acute angle to the out-lever (HR). The mandible of Italian pygmy shrews, in comparison to other European pygmy shrews, was characterized by a different slope of the incisive alveolus and a bigger ratio of the coronoid process versus the horizontal ramus lengths leading to an increased bite force, and by substantial changes in the posterior portion that created a more acute angle between the condyloid process and the horizontal ramus (Fig. 5B, C). The greater bite force of Italian pygmy shrews compared to other European pygmy shrews is likely a consequence of adaptation to the more arid conditions and prey with harder exoskeletons (Strait, 1993; Carraway & Verts, 1994). An alternative hypothesis is that inter- or intra-specific competition might cause an increase in mandible size and bite strength (Corti & Rohlf, 2001). However, considering the low population densities of pygmy shrews in southern Italy and the similarity of species assemblages throughout the animal's range, this hypothesis appears unlikely.

Refugia within refugia in the Italian peninsula?

One possible explanation for the current occurrence of two morphologically and genetically distinct clusters of pygmy shrew essentially restricted to Italy is that there were two glacial refugia for this species in this region, and that these distinct groups arose within those refugia during one or more glacial cycles. Thus, for the pygmy shrew, the Italian refugial area may have been subdivided into multiple refugia at the LGM, concordant with the 'refugia within refugia' concept (Gómez & Lunt, 2006). However, genetic subdivision may also arise from population subdivision at times other than glacial maxima.

One of the two groups of pygmy shrews in Italy is very widespread (the Italian group), whereas the other is limited to the extreme south (the southern Italian group). It is apparent that a variety of other small vertebrates are also characterized by a genetic lineage in the extreme south of Italy (Table 1) and, in most of

Table 1. Small vertebrate species with a distinct genetic lineage in the southern part of the Italian peninsula

Class	Species	Distribution range of species	Southern Italian lineage	Reference
Amphibia	*Triturus italicus*	Central–southern Italy	Calabria	Ragghianti & Wake (1986)
	Rana (*Pelophylax*) *lessonae*	Europe	Calabria and Sicily	Santucci *et al*. (1996); Canestrelli & Nascetti (2008)
	Salamandra salamandra	Europe	Southern Italy	Steinfartz, Veith & Tautz (2000)
	Salamandrina terdigitata	Central–southern Italy	Southern Italy	Mattoccia, Romano & Sbordoni (2005); Nascetti, Zangari & Canestrelli (2005); Canestrelli, Zangari & Nascetti (2006a)
	Bombina pachypus	Peninsular Italy	Calabria	Canestrelli *et al*. (2006b)
	Hyla intermedia	Peninsular Italy and Sicily	Calabria and Sicily	Canestrelli *et al*. (2007)
	Rana italica	Peninsular Italy	Calabria	Canestrelli *et al*. (2008)
Reptilia	*Hierophis viridiflavus*	Europe	Calabria and Sicily	Nagy *et al*. (2003)
	Podarcis sicula	Italy & Balkans	Calabria	Podnar, Mayer & Tvrtkovic (2005)
	Vipera aspis	Europe	Southern Italy	Ursenbacher *et al*. (2006)
	Lacerta bilineata	Europe	Calabria	Böhme *et al*. (2007)
Mammalia	*Lepus corsicanus*	Central–southern Italy	Southern Italy and Sicily	Pierpaoli *et al*. (1999)
	Talpa romana	Central–southern Italy	Calabria	Ungaro *et al*. (2001)
	Myodes glareolus	Eurasia	Calabria	Amori *et al*. (2008)
	Microtus brachycercus	Central–southern Italy	Central–southern Italy	Castiglia *et al*. (2008)
	Sciurus vulgaris	Eurasia	Southern Italy	Grill *et al*. (2009)
	Sorex minutus	Eurasia	Southern Italy	Present study

these examples, there is at least one more lineage restricted to Italy (a second, Italian-restricted lineage has not been recorded in *Salamandra salamandra*, *Hierophis viridiflavus*, *Lacerta bilineata* or *Sciurus vulgaris*). So, in the pygmy shrew, as in a range of other species, this may be the result of one glacial refugium in the extreme south of Italy, and at least one other refugium further north. In *Rana* (*Pelophylax*) *lessonae*, *Vipera aspis* and *Myodes glareolus*, there is a similar situation as in the pygmy shrew, with an Italian lineage that makes contact with one or more other European lineages in the extreme north of Italy (Santucci, Nascetti & Bullini, 1996; Deffontaine *et al*., 2005; Ursenbacher *et al*., 2006; Canestrelli & Nascetti, 2008). For each of these species, the widespread Italian lineage may be presumed to derive from a glacial refugium located somewhere within the vicinity of the Apennine mountain chain.

Although the data appear consistent with the 'refugia within refugia' concept, a degree of caution is needed for the pygmy shrew, and probably for some other species as well. The concept implies that the species were restricted to particular localized areas at the LGM, which can be termed 'refugia' (Bennett & Provan, 2008; Stewart *et al*., 2010), and that their distribution expanded on amelioration of the climate. Indeed, the pygmy shrew is currently restricted to high altitude areas in Italy because the dry, hot Mediterranean conditions in the lowlands do not suit it. Therefore, in southern Europe, the distribution of the pygmy shrew may actually be more restricted during interglacials than glacials, in line with ideas of 'interglacial refugia' (Hilbert, Graham & Hopkins, 2007).

Further samples are needed to test whether the 'refugia within refugia' concept applies to the pygmy shrew in Italy (i.e. genetic analyses are needed to demonstrate population expansions from two separate refugial areas). From the current data, it is possible that the Italian and southern Italian morphological and genetic clusters occupied a single, continuous area at the LGM with the integrity of the groups retained by a hybrid zone (Barton & Hewitt, 1985).

Thus, pygmy shrews from southern Italy are genetically and morphologically distinctive, and may

be restricted to Calabria. The general question that remains is: how did the southern Italian and Italian groups of pygmy shrew become separate entities? Here, comparison with other small vertebrates can be informative. For many of the species with a southern Italian lineage, that lineage is also restricted to the peninsula of Calabria (Fig. 1A, Table 1). Calabria consists of isolated mountain massifs separated by lowland areas. From the Pliocene to the Middle Pleistocene, at times of high sea level, those massifs would have been islands in a chain that stretched between the southernmost part of the Italian peninsula and Sicily (Malatesta, 1985; Caloi, Malatesta & Palombo, 1989; Bonardi *et al*., 2001; Bonfiglio *et al*., 2002). Since the Late Pleistocene, uplift has prevented island-formation. Considering all species that have distinct Calabrian lineages, it is reasonable to suggest that the distinction of these Calabrian forms reflects island evolution. On the basis of the molecular clock used in the present study, the separation of the Italian and southern Italian cyt *b* lineages of pygmy shrew from each other and from the European lineage extend back into the Middle Pleistocene.

Over the last glacial cycle, the putative Calabrian island forms have been limited to a mainland distribution. For the pygmy shrew at least, it is likely that the Calabrian form is geographically isolated at the present time because pygmy shrews there are apparently restricted to the Calabrian massifs. The extent to which Calabrian pygmy shrews were isolated during the period extending back to the LGM is less certain, and is of course crucial in relation to the 'refugia within refugia' concept.

Calabria forms part of the 'Calabrian Arc' and includes the separate mountain massifs of Catena Costiera in the north, La Sila in central Calabria, and the Serre and Aspromonte massifs in southern Calabria (Fig. 1A; Bonardi *et al*., 2001). Catena Costiera makes contact in the north with the southern Apennines (southern Lucania, Basilicata region) but it is separated from La Sila by the Crati Valley depression in the east and from southern Calabria by the Catanzaro Plain in the south. Therefore, pygmy shrews from Catena Costiera in the northern Calabrian Arc might have been able to remain in long-term contact with the southern Apennine populations, thus avoiding differentiation. This could explain why the morphological samples from Catena Costiera (I10) clustered with northern–central Italian and not with the geographically nearby southern Italian samples from La Sila (I11) and Aspromonte (I12). For the southern Italian pygmy shrew, the La Sila and Aspromonte massifs are geographically isolated from each other and from the Apennines.

Taxonomic and local conservation implications for southern Italian pygmy shrews

The subspecies concept has been widely criticised (Wilson & Brown, 1953), although the use of trinomials can generate interest at a local scale by giving a regional- or country-based value for biodiversity, and the recognition of subspecific levels may stimulate further investigation of those populations (Mayr, 1982; Amori, Angelici & Boitani, 1999). The morphological and genetic differentiation shown in the present study does not justify that Italian and/or southern Italian pygmy shrews be considered as separate species. However, we consider that the trinomial *S. m. lucanius* should be kept as the taxonomic name to describe the southern Italian pygmy shrews, emphasizing that it is an Evolutionarily Significant Unit (ESU; Moritz, 1994). Our data also suggest that Italian pygmy shrews form a different ESU from the European *S. m. minutus*. Given that the Italian and southern Italian pygmy shrews are distinctive forms, do they need protection? Currently, the pygmy shrew overall is characterized as a Least Concern species for conservation purposes (IUCN, 2007; http://www.iucnredlist.org/details/29667/0). However, the Italian populations deserve more attention than this implies. In particular, the distinctive Calabrian populations of pygmy shrew surely need specific protection. They occur in a very small area and, on the basis of our field efforts, are very uncommon there (R. Vega & G. Aloise, pers. observ.). Given the range of species and populations that are distinctive in Calabria (Table 1), there needs to be a clear conservation effort to protect plant and animal communities in that region.

CONCLUSIONS

On the basis of the morphological and phylogeographic data presented here, we show three distinguishable and significantly differentiated clusters of pygmy shrews corresponding to European, Italian, and southern Italian groups. The differentiation includes adaptive features, on the basis of our analysis of mandible characteristics. The two Italian groups appear to have arisen as a consequence of geographic isolation before the LGM (one group originating on islands in the location of present-day Calabria), and it is unclear whether they were located in separate glacial refugia at the LGM. Moreover, because studies of several other species have also shown genetic and morphological distinction of populations in Calabria, this region should be a focus for conservation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Samples and localities for phylogeographic analysis of pygmy shrews.
Table S2. List of primers used for the amplification of cytochrome *b* (cyt *b*) and Y-chromosome introns from pygmy shrews.
Table S3. Samples and localities for morphological analysis of pygmy shrews.

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