Introductions over introductions: the genomic adulteration of an early genetically valuable alien species in the United Kingdom.

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ABSTRACT. Invasive alien species are a major cause of biodiversity loss. Nevertheless, non-native species can also contribute to conservation objectives. In 1673, the red-legged partridge (*Alectoris rufa*), a galliform native to southwest Europe, was introduced from France (*A. r. rufa*) into the UK for hunting purposes. Nowadays, hunters constantly supplement natural populations of *A. rufa* in its native range with stocks of captive-bred individuals. Such birds are usually genetically unscreened, and human-mediated hybridization with the exotic chukar (*Alectoris chukar*) has undermined genomic integrity of the species. *Alectoris rufa* in the UK has never been genetically investigated, and birds from East Anglian estates with no modern history of supplementation offer a potential genomic backup for the highly polluted native-range *A. r. rufa*. We genotyped modern and ancient (1824-1934) birds at the mitochondrial DNA (mtDNA) level to determine present and past kinship between East Anglian and native-range *A. rufa*. We used Short Tandem Repeats (STR) and Random Amplified Polymorphic DNA (RAPD) markers to identify *A. rufa* x *A. chukar* hybrids. The kinship of East Anglian birds with *A. r. rufa* was confirmed. No *A. chukar* introgression was found in ancient East Anglian *A. rufa*. Among modern partridges, we found birds with *A. chukar* mtDNA, and both STRs and RAPDs disclosed many *A. rufa* x *A. chukar* hybrids. While the genetic analysis pointed to the increase of diversity and decline of disparity over time within and among *A. rufa* populations, respectively, the conservation value of the resource historically introduced to the UK proved to have been quashed by three decades of recent releases of *A. chukar* and its *A. rufa* hybrids.

Key words: *Alectoris chukar*, *Alectoris rufa*, anthropogenic hybridization, biotic homogenization, exotics, galliforms.

INTRODUCTION

Invasive alien species are one of the major causes of loss of biodiversity (e.g.: Mack et al. 2000, Clavero and García-Berthou 2005). Their recent proliferation is mostly due to the global transportation that is moving species around like never before, a process that tends to minimize biological distinction among regions through the gradual replacement of native biotas by locally expanding non-natives ("biotic homogenization": Olden et al. 2004).

Conservationists work hard to mitigate serious problems caused by alien species. Nevertheless, the potential conservation value of non-natives has been recently highlighted. In some cases alien species can provide habitat, shelter and food to native ones, catalyze restoration of indigenous communities, recover ecological services by replacing the role of extinct taxa, and contribute to ecosystem resilience and stability through the increase of local species richness (Salm et al. 2009; Griffith et al. 2010; Schlaepfer et al. 2011, 2012). Davis et al. (2011) claimed for *ad hoc* assessment of the environmental impact of any given taxon rather than judging the same merely on whether it can be native or alien. Many scientists questioned such a potentially new perspective stressing that even the smallest lowering of the guard against aliens could be not justified (e.g.: Alyokhin 2011, Lerdau and Wickham 2011, Lockwood et al. 2011, Simberloff 2011).

Human-driven foundation of animal populations does not necessarily result in erosion or depletion of the original genetic variability of the species involved. Under some circumstances, introductions contributed to preserve viable populations of species threatened by extinction. For instance, a wild and genetically pure population of the endangered banteng (*Bos javanicus*) translocated from Indonesia into Australia has saved the species from hybridization with other bovids (Bradshaw et al. 2006). Similarly, the fallow deer (*Dama dama*) population introduced a few thousands of years ago into the island of Rhodes (Greece),

73 shows a remarkable portion of the original genetic diversity of the native Anatolian source,
74 which by contrast is seriously threatened (Masseti et al. 2008).
75 The red-legged partridge (*Alectoris rufa*, Phasianidae) (RLP) is a medium-sized galliform
76 hunted throughout its entire native range (Iberian Peninsula, France and Italy). Three
77 subspecies are recognized based on subtle morphological differences: *A. r. hispanica*
78 (northern and central Portugal, north-western Spain), *A. r. intercedens* (southern and eastern
79 Spain, Balearics included), and *A. r. rufa* (Italy and France, Corsica included) (Madge and
80 McGowan 2002). However, given that these subspecies are largely contentious, we prefer to
81 refer herein to them as populations. The RLP is in such high demand by hunters that
82 commercial stocks of captive-bred individuals constantly supplement natural populations.
83 Hybridization with the exotic chukar partridge (*A. chukar*: Greek islands, Cyprus, and from
84 Middle East to East Asia) has been documented across the whole range of *A. rufa* because of
85 releases to the wild of farm-bred *A. rufa* x *A. chukar* individuals. In captivity the chukar is the
86 most prolific *Alectoris* breeder, and when crossed with RLPs, the offspring are equally
87 prolific. Such an intensive management has led to the virtual loss of the native genome of the
88 *A. r. rufa* subspecies, which is the most heavily genetically polluted race (Dias 1992; Baratti
89 et al. 2004; Barbanera et al. 2005, 2009a, 2011; Barilani et al. 2007; Tejedor et al. 2007;
90 Blanco-Aguiar et al. 2008; Martínez-Fresno et al. 2008; Casas et al. 2012; Negri et al. 2013).
91 The United Kingdom (UK) harbours an introduced *A. r. rufa* population. In 1673, King
92 Charles II introduced the RLP to the UK for hunting purposes. These birds came from
93 Chambord in the Loire valley (Loire-et-Cher Department, France) and were released in
94 Windsor Great Park, on the border between Berkshire and Surrey (Lever 1977, Potts 2012).
95 After several more releases in the area the species was well established in East Anglia by
96 around 1790. Since then the species has increased in abundance in the UK, currently

numbering around 82,000 (Musgrove et al. 2013). The RLP mostly ranges from southern, south-eastern and eastern England northwards to Yorkshire. It has been the subject of many studies, ranging from its ecology through to population dynamics and behaviour (e.g.: Middleton and Chitty 1937; Blank and Ash 1955; Potts 1980; Green 1983, 1984; Rands 1986; Aebischer and Potts 1994; Aebischer and Lucio 1997; Tompkins et al. 2002). From the 1960s, commercial game farms began importing the chukar and rearing its crosses with *A. rufa* for subsequent release in UK (Potts 1989). Mounting concern over the negative impact of these releases on the reproduction of *A. rufa* led to a ban on the releasing of *A. chukar* or its hybrids in 1992 (Goodwin 1986; Potts 1989, 1991; Payn 1991).

The UK *A. rufa* population has never been genetically investigated, and parts of it could potentially represent an ancient genomic backup for the native yet polluted *A. r. rufa*. We investigate *A. r. rufa* in East Anglia in an attempt to identify remnant populations of high genetic value for the conservation management of this taxon. We use mitochondrial DNA (mtDNA) to infer kinship with modern *A. rufa*, and we attempt to identify potentially occurring *A. rufa* x *A. chukar* hybrids using markers relying on either probabilistic (microsatellites or Short Tandem Repeats, STR) or categorical (Random Amplified Polymorphic DNA, RAPD) assessment. In order to investigate the relationships between introduced and native *A. rufa* in a temporal framework, we analyse the mtDNA genotype of ancient (1824-1915) East Anglian *A. rufa*, and compare them to the mtDNA RLP (1856-1934) dataset of Barbanera et al. (2010).

METHODS

Sampling was carried out by Norfolk gamekeepers and staff of the Game & Wildlife Conservation Trust during the 2010 hunting season. We focused on an area of about 180 km^2

121 located between latitude 52°96' - 52°34' North and longitude 0°35' - 0°81' East in the county
122 of Norfolk, East Anglia (Fig. 1). This region of predominantly arable farmland was chosen
123 because it is the wild partridge stronghold of the UK, both for *A. rufa* and grey partridges
124 *Perdix perdix* (Balmer et al. 2013). Several estates there deliberately manage their wild
125 partridge stocks for traditional driven shooting and have no known history of releasing
126 captive-bred birds (although neighbouring estates may release). We obtained samples for
127 genetic analysis from birds shot at such sites during driven shoot days. Samples from the
128 same site were all collected on the same shoot day, but the numbers available were large and
129 the birds were sampled at random. A single toe-pad was cut from the right foot of each bird (*n*
130 = 58) and preserved in 96% ethanol. We also used *A. rufa* samples (blood, feather, liver: *n* =
131 153) collected in the Iberian Peninsula, France (with Corsica) and Italy, i.e. those used in
132 Barbanera et al. (2011), plus six additional Portuguese RLPs (*n* = 58 + 153 + 6 = 217, total
133 sample size) (Fig. 1, Table S1).
134
135 **Biological sampling: ancient RLPs**
136 We acquired slivers of toe pads from 10 specimens of East Anglian RLPs held in the Natural
137 History Museum (Tring, UK) and collected between 1824 and 1915, well before *A. rufa*
138 supplementary stocking became common or *A. chukar* was introduced to the UK. We also
139 took samples from three specimens of melanistic *A. rufa* from Essex and Kent (Table S1), as
140 Soland (1861) described melanistic RLPs from the Loire valley (France), where the stock
141 originally imported to UK in 1673 came from (see above). We compared these RLPs to 47 *A.*
142 *rufa* samples (dated 1856 - 1934) provided by several European and US museums, and
143 collected across the entire range of the species (Barbanera et al. 2010). We augmented the
144 sample size with seven additional (*n* = 13 + 47 + 7 = 67) French *A. rufa* samples (1872-1938)

provided by the Natural History Museum at Tring (Table S1).

Modern DNA extraction

We conducted all modern DNA extractions at the Department of Biology (Zoology and Anthropology Unit - Zoology building). We extracted DNA from liver and blood using the Puregene Core Kit-A (Qiagen) following the manufacturer's instructions, and from feathers as in Barbanera et al. (2005).

Ancient DNA extraction

Twenty *A. rufa* specimens (England, n = 13; France, n = 7: Table S1) were investigated in Pisa at the Department of Biology (Zoology and Anthropology Unit - Anthropology building). The QIAamp DNA micro kit (Qiagen) was employed to extract DNA from a small amount of tissue (≤ 5 mg) cut into tiny pieces using a sterile, single-use razor blade (BBraun, Aesculap Division). Fragments were processed in compliance with the manufacturer's instructions, with the following differences in case of very hard tissues: (i) incubation in the water bath up to 48 h; (ii) addition of 3 μL of dithiothreitol (100 mg/ml); (iii) double amount of proteinase K (20 mg/ml). DNA isolation and all pre-PCR work were performed in a dedicated facility strictly adhering to ancient DNA protocols at all stages.

Modern mitochondrial DNA

We amplified 1092 bp of Cyt-*b* from 58 (East Anglia) and six (Portugal) modern *A. rufa*. A mix including 20 ng of template DNA, 1 μL of Ampli*Taq* Gold DNA Polymerase (1 U/μL, Applied Biosystems), 4 μL 25 mM $MgCl_2$ (Applied Biosystems), 5 μL of PCR Gold buffer (Applied Biosystems), 5 μL 2.5 mM dNTP (Sigma Aldrich), and 3 μL of each primer (final

concentration, 1 μM) was added to each reaction tube (volume, 50 μL). Thermal profile was: 10 min at 94 °C; then, 30 cycles of 1 min at 94 °C, 2 min at 55 °C, and 1 min at 72 °C; final extension, 72 °C for 7 min. We purified PCR products using the GenElute PCR Clean-up Kit (volume, 40 μL; Sigma Aldrich). The sequencing was performed in both directions on an ABI 3730 DNA automated sequencer at Genechron (Rome, Italy). We screened out all *A. chukar* mtDNA sequences (see Results) and aligned the *A. rufa* ones twice with CLUSTALW (v. 1.81: Thompson et al. 1994). The first time we used the entire 1092-bp fragment, while in the second we selected the 229-bp portion shared with the museum samples (see below). In both alignments we included all the *A. rufa* entries of Barbanera et al. (2011) (Table S1).

We computed haplotype composition and diversity ($h \pm$ SD) using DNASP (v. 5.00: Librado and Rozas 2009). We investigated the partition of genetic diversity among and within populations by AMOVA with ARLEQUIN (v. 3.5.1: Excoffier and Lischer 2010) using ϕ_{ST} pairwise distance analogous to Wright's (1951) *F*-statistics (1000 permutations). We plotted these values on the first two axes of a Principal Components Analysis (PCA) using STATISTICA v. 5.0/W (Statsoft Inc., USA). We also constructed a haplotype network with the Median Joining method of Bandelt et al. (1999) using NETWORK v. 4.5.1.0 (Fluxus Technology).

In the event of *A. chukar* maternally-introgressed RLP, we also sequenced the entire Control Region (CR) gene of the mtDNA as in Barbanera et al. (2005), and compared joined sequences (Cyt-*b* + CR) to the chukar dataset of Barbanera et al. (2009a) created by using 179 samples collected from the native range (18 countries: from Greece to China) and from the range of chukars introduced to North America (USA) (106 haplotypes, GenBank codes: AM850718-786, AM850791, AM850793-828). We aligned *A. chukar* Cyt-*b* + CR sequences with CLUSTALW and inferred haplotype composition using DNASP. We computed ϕ_{ST} average

distance values among *A. chukar* populations with ARLEQUIN (1000 permutations) and plotted these values on the first two axes of a PCA using STATISTICA.

Ancient mitochondrial DNA

We amplified 229 bp of Cyt-*b* (pos. 358-586 of Z48775 GenBank sequence: Randi 1996) from 20 *A. rufa* museum specimens. Different to Barbanera et al. (2010) we used primers AR3a-1 (5′-CCT GCT CCT CAC ACT AAT AGC C-3′) and AR3a-2 (5′-TGA TGG TAA TTC CTG CGA TT-3′) in a single PCR with the following thermal profile: 10 min at 94° C; then, 50 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 45 s; final extension, 72 °C for 7 min. Using CLUSTALW, we aligned the 229-bp Cyt-*b* sequences with those obtained from 47 museum RLPs by Barbanera et al. (2010) (Table S1). PCR product purification and direct sequencing as well as all the mtDNA population genetic computations (with the exception of the network) were done as for the modern *A. rufa*.

Microsatellite DNA

We genotyped all 58 partridges from East Anglia at seven STR loci (Table 1). Three loci (MCW104, MCW118 and MCW280) were originally isolated from domestic chicken (*Gallus gallus*) at the Wageningen University (The Netherlands), while the other ones from the RLP genome by Gonzalez et al. (2005) (Aru1.23, Aru1.27) and Ferrero et al. (2007) (Aru1E45, Aru1I68). We also used analogous STR profiles of genetically homogeneous *A. rufa* (n = 30, Spain) and *A. chukar* (n = 30: Greece, Cyprus) (Barbanera et al. 2010) as reference to test the East Anglian RLP population for the occurrence of hybrids between the two species. PCR conditions were as in Barbanera et al. (2009b).

The discriminatory power of the whole set of STR loci was evaluated with GIMLET (v. 1.3.3: Valière et al. 2002) by estimating the probability that two individuals drawn at random from the populations showed identical multilocus genotypes by chance (P_{ID} and P_{ID}sib: for the latter, we assumed sibling relationships) (Paetkau et al. 1998, Waits et al. 2001). We used GENEPOP (v. 3.4: Raymond and Rousset 1995) and ARLEQUIN to calculate expected (H_E) and observed (H_O) heterozygosity, and to infer deviations from Hardy-Weinberg Equilibrium (HWE) and Linkage Disequilibrium (LE). The significance level of HWE and LE tests was estimated using the Bonferroni correction (Hochberg 1988).

Bayesian clustering analysis was performed with the program STRUCTURE (v. 2.3.4: Pritchard et al. 2000) (admixture model with independent allele frequencies among populations) to investigate the posterior probability of each specimen belonging to one parental species or having fractions (Q_i) of its genome originating from two parental species. All simulations were run with 10^6 iterations, following a burn-in period of 10^5 iterations, and replicated four times per each *K*-value. We assumed that the maximum number of populations (*K*) varied between 1 and 8, and calculated the optimal *K* value and the identification threshold ($Q_i = 0.90$; cf., Vaha and Primmer 2006) to assign each individual to one or jointly to two clusters as in Barbanera et al. (2009b).

Random Amplified Polymorphic DNA

We genotyped all 58 East Anglian partridges using *A. rufa vs. A. chukar* species-specific RAPD markers (OP-C-08, OP-C-09, OP-C-20, OP-H-12: Operon Technologies Inc.) of Barbanera et al. (2005). These primers worked reliably to disclose either *A. chukar*-introgressive hybridization into *A. rufa* (Barbanera et al. 2005, 2009a, 2010) or the opposite as well (Barbanera et al. 2007). We used the protocol of Barbanera et al. (2005) and the parental

controls (*A. rufa*, *n* = 30; *A. chukar*, *n* = 75) of Barbanera et al. (2010). The concurrent presence of distinctive *A. rufa* and *A. chukar* bands in the RAPD profile of a given specimen was considered occurrence of *A. rufa* x *A. chukar* hybridization. Each individual was tested three times for each primer. Bands were scored only when present in all replicates.

RESULTS

Modern mtDNA

Ten East Anglian RLPs showed *A. chukar* mtDNA lineage while the remaining ones (*n* = 48, 83%: Table S2) followed the *A. rufa* maternal line. All six individuals from Portugal showed *A. rufa* mtDNA lineage. Hence, modern (1092 bp, Cyt-*b*) *A. rufa* alignment included (*n* = 48 + 6 +153 =) 207 sequences. We found 33 haplotypes (mH; GenBank codes: HG940431- 63). Eleven were disclosed in the East Anglian population: eight (73%) were private haplotypes and three (mH1, mH3, mH10) common to all investigated populations (Fig. 2). The Spanish *A. rufa* samples showed a percentage of private haplotypes similar to that of England. The Spanish and Corsican populations showed the highest and lowest diversity value, respectively (Table 2). The AMOVA (229 bp, Cyt-*b*) showed that 45.6% of the total genetic variability was partitioned among populations, the remaining 54.4% within them (ϕ_{ST} = 0.46, $P < 0.001$). When the Corsican population was removed, the percentage value changed to 16.6% (among populations) and 83.4% (within populations) (ϕ_{ST} = 0.17, $P < 0.001$), respectively (Table 3).

Cyt-*b* (1092 bp) + CR (1154 bp) combined *A. chukar*-like mtDNA sequences (2246 bp) disclosed in East Anglian RLPs (*n* = 10) showed three haplotypes. They were identical to *A. chukar* haplotypes named C1 (*n* = 2), C69 (*n* = 5) and C73 (*n* = 3) in Barbanera et al. (2009a), and deposited in the GenBank with codes AM850718, -786 and -790, respectively.

When the ϕ_{ST} pairwise distance values among all chukar populations were plotted on a PCA, the first two axes explained 78.5% of the total diversity. The *A. chukar*-like mtDNA sequences of the phenotypic RLPs from East Anglia (Fig. 4) clustered with those from both the easternmost part of the *A. chukar* native distribution range (China) and the USA.

Ancient mtDNA

We found only *A. rufa* mtDNA lineage in the ancient RLPs. Museum specimens from East Anglia (n = 13) and France (n = 7) showed the same haplotype (GenBank code: FN547893; Table S1). Genetic diversity increased when moving from East (h = 0: Italy and Corsica) to West (h = 0.73 ± 0.15: Portugal) across the native range of the species (Table 3). The AMOVA showed that 21.6% of the total genetic variability was partitioned among populations, the remaining 78.4% within them (ϕ_{ST} = 0.22, P < 0.001). When the Corsican population was removed from the analysis, the percentage value changed slightly to 22.3% (among populations) and 77.7% (within populations) (ϕ_{ST}, as above), respectively (Table 3).

Modern *versus* ancient mtDNA

When the ϕ_{ST} pairwise distance values (229 bp, Cyt-*b*: only *A. rufa* mtDNA lineage) among modern and ancient RLP populations were plotted on a joint PCA, the first two axes explained 89.1% of the total diversity (Fig. 3). The Corsican modern population was the most diverging one. The latter excluded, (i) all modern RLPs (black circles) clustered together and were sharply separated from all ancient ones (white circles), and (ii) the percentage of the total genetic variability that was partitioned among populations decreased from 22.3 to 16.6 (ϕ_{ST}, from 0.22 to 0.17, respectively, all P < 0.001: Table 3). In the PCA, modern RLPs stretched for *c*. 0.3 and 0.6 units along the first and second component, respectively. Ancient

RLPs scattered within a span of *c.* 1.0 and 0.8 units along the first and second component, respectively, and showed a clinal distribution of populations across the *A. rufa* range.

Microsatellite DNA

In the entire sample size (n = 58 + 30 + 30 = 118) the STR panel was powerful in discriminating individuals ($P_{ID} = 1.54 \times 10^{-7}$ and $P_{ID}sib = 1.02 \times 10^{-7}$), as values lower than 0.001 are considered as satisfactory (Waits et al. 2001). Locus MCW118 was monomorphic in both *A. rufa* and *A. chukar* control samples, whereas only in the latter species loci MCW280 and Aru1I68 were monomorphic (Table 1). In the RLPs of East Anglia we found a few private alleles (n = 3) while a higher number was disclosed in the *A. rufa* (n = 11) and *A. chukar* (n = 8) parental controls. The population of East Anglia showed an overall significant departure from the HWE (Fisher test, $P < 0.001$, Table 1) only due to heterozygote deficiency in loci MCW280 and Aru1I68. The LE test carried out for all pairs of loci did not disclose any linked loci (data not shown). Bayesian clustering analysis compared the East Anglian population to *A. rufa* and *A. chukar* controls (K = 2; clusters I and II, respectively, Fig. 5). Individual membership (Qi) revealed that there was no allele admixture between parental species and no birds with Qi < 0.90 were found. However, a high degree of genetic admixture was found in the East Anglian population, and only 27 individuals could be assigned to cluster *A. rufa* with Qi (I) > 0.90. The remaining 31 showed admixed genotype of different degree between *A. rufa* and *A. chukar* parental species (Table S2): the average membership probabilities obtained for this population were Qi (I) = 0.88 and Qi (II) = 0.12.

Random Amplified Polymorphic DNA

The RAPD banding profile of four East Anglian RLPs, six *A. rufa* and six *A. chukar* controls is given in Fig. S1: all birds were identified as *A. rufa* x *A. chukar* hybrids. Overall, no birds were found to be hybrid for all primers; three (no. 5, 14 and 43: Table S2) showed only the *A. rufa* band, the remaining ones were *A. rufa* x *A. chukar* hybrids with intermediate pattern.

The overall genetic profile (mtDNA, STR, RAPD) for all East Anglian RLPs is reported in Table S2. Overall, 94% of East Anglian birds were hybrid. Only three partridges (no. 5, 14 and 43) showed no sign of *A. chukar* genes. MtDNA, microsatellite DNA and RAPD markers showed an increasing *A. chukar*-introgressed RLP detection power (17%, 53% and 94%, respectively).

DISCUSSION

For long time conservation professionals have been emphasizing the harmful effects of invasive alien species on native biotas (e.g., Rhymer and Simberloff 1996; Mooney and Hobbs 2000; Lodge et al. 2006). Nevertheless, after the recent review entitled "The Potential Conservation Value of Non-Native Species for Conservation" (Schlaepfer et al. 2011), a more balanced view on alien species has come to the fore (see, on one side, e.g.: Davis et al. 2011, Schlaepfer et al. 2012; on the other, e.g.: Simberloff 2011, Vitule et al. 2012). Our work fits into this context, because we sought to establish whether the XVIIth century introduction of *A. rufa* to the UK provided, albeit not deliberately, a useful genomic backup for the *A. r. rufa* populations. The latter is threatened with genetic extinction (*sensu* Rhymer and Simberloff 1996) owing to anthropogenic hybridization with the chukar (*A. chukar*). The non-native UK population could represent an important resource for the genetic conservation of *A. r. rufa* in its native range.

333 Since the papers of Crandall et al. (2000) and Fraser and Bernatchez (2001), the
334 importance of adaptive evolutionary conservation has been comprehensively recognized. A
335 given resource should be evaluated over a temporal and spatial framework to properly infer its
336 conservation value. This is even more important when phylogeographic investigations are
337 carried out in game species subjected to intense *ex situ* management. We confirmed the tight
338 genetic kinship between historical and modern *A. rufa* populations in East Anglia with
339 populations from the easternmost part of the species' range (Fig. 3). Whereas no sign of *A.*
340 *chukar* introgression was found in the ancient RLPs, we found mixed maternal ancestry in
341 modern ones, with 17% of birds showing mtDNA haplotypes discordant with their *A. rufa*
342 phenotype and corresponding to that of *A. chukar*. STRs and RAPDs disclosed a high
343 percentage (94%) of modern *A. rufa* x *A. chukar* hybrids (Table S2).
344
345 **The East Anglian population within the genetic structure of the *A. rufa* species**
346 Modern East Anglian RLPs showed three haplotypes (mH1, mH3, mH10: Fig. 2) largely
347 shared with all populations from the native *A. rufa* range. This might be due to the release in
348 the wild of farm-bred individuals of different geographic origin, although no definitive proof
349 is available. Yet East Anglian RLPs also showed a high percentage of private haplotypes
350 among the studied populations (Fig. 2, Table 2), suggesting the persistence of genetic material
351 from the original introductions.
352 Genetic information from ancient East Anglian RLPs confirmed their tight relationship
353 with *A. rufa rufa*. However, different to what was reported by Lever (1977) and Potts (2012),
354 they were closer to the Italian and Corsican conspecifics than to those from continental France
355 (Fig. 3: white circles). Melanistic RLPs did not diverge with respect to the others sampled in
356 England and held in the same museum collection (Table S1). Although this outcome could be

357 a drawback conveyed by the use of just a portion of a single mtDNA gene, we could not
358 either establish or refute a direct connection between *A. rufa* from East Anglia and *A. rufa*
359 from the Loire valley (France), as seemed likely according to Soland (1861).
360 When ancient and modern *A. rufa* were compared, we found two opposite trends. On one
361 hand, intra-population divergence (haplotype diversity, *h*) increased over time across the *A.*
362 *rufa* range, England included (Table 2). This would seem the result of intense *ex situ*
363 management (i.e., selection in captivity) and subsequent releases in the wild as suggested by
364 Rodríguez García and Galián (2014). Recently, Casas et al. (2012) proved for the first time in
365 the wild that hybrid females have the same probability of laying a clutch (albeit a larger one),
366 and similar hatching success, as "pure" ones, i.e. that hybrid females can spread their admixed
367 genotypes efficiently despite their lower survival. On the other hand, inter-population
368 divergence (pairwise distance, ϕ_{ST}) decreased over time (Fig. 3) across the *A. rufa* range,
369 England included. In the past (Fig. 3: white circles), *A. rufa* populations of Italy and Corsica
370 (with England) diverged from those inhabiting the western part of the species' range (Spain
371 and Portugal), the French one lying in between (Fig. 3). Remarkably, this ancient *A. rufa*
372 genetic pattern mirrored the clinal distribution of known subspecies across the *A. rufa* range
373 (Madge and McGowan 2002). At the present time (Fig. 3: black circles), for instance, there is
374 no significant differentiation among Italian, French and Portuguese *A. rufa* (ϕ_{ST} data, not
375 shown), and all modern RLPs clustered much more closely each other than the ancient ones.
376 In conclusion, while the genetic diversity (*h*) has increased within each population likely
377 because of releases of not-local captive-bred RLPs, the latter has led genetic disparity (*sensu*
378 Gould 1989) among populations (ϕ_{ST}) to decline over time within the species *A. rufa*.
379 Altogether this points to the occurrence of genetic homogenization within the species (Olden
380 et al. 2004), as already showed in the paper of Rodríguez García and Galián (2014, p. 62)

where restocked populations were also sampled. When populations with diagnosed *A. rufa* hybrids were excluded from the analysis, as in Ferrero et al. (2011, p. 2634), a remarkable phylogeographic structure was proved still to occur in the species.

Hybridization in the East Anglian *A. rufa* population

Alectoris rufa x *A. chukar* birds have been observed in the wild in the UK (e.g.: Lever 1987, Wilkinson 1987). Our genetic analysis has confirmed that hybrids are still very much present in the UK. Unfortunately, this is true of East Anglia, where we believed that we had the strongest chance of finding pure *A. rufa*. We used DNA markers relying on either categorical (mtDNA, RAPD) or probabilistic (STR) assessment to disclose hybrids. Nevertheless, nuclear DNA markers are those strictly deputed to accomplish such an experimental task, uniparental mtDNA being able to provide indications yet not definitive proofs. Among the 17% of East Anglian RLPs holding *A. chukar* mtDNA, none showed an *A. rufa* genotypic profile when tested by STRs and RAPDs (Table S2). Conversely, almost all East Anglian RLPs showing *A. rufa* mtDNA were disclosed to be real *A. rufa* x *A. chukar* hybrids by STRs and RAPDs. Furthermore, as in Barbanera et al. (2009a), we proved that well-established species-specific RAPDs can disclose hybrid partridges not only without the probabilistic assessment provided by the Bayesian statistics but also more efficiently than STRs (detected hybrids: 94% by four RAPDs *versus* 65% by seven STRs, respectively: Fig. 5, Fig. S1 and Table S2). Although the sources in Norfolk were known to have no modern history of restocking with farm-bred RLPs, hybridization would have come from neighbouring areas where supplementation with genetically unscreened birds did occur (cf., Blanco-Aguiar et al. 2008).

We found tight genetic closeness between the chukar haplotypes disclosed in the East Anglian RLPs and those held by *A. chukar* native to China or introduced to USA (Fig. 4).

With regards to the latter, it is well documented that the founders belonged to a mix of East Asian subspecies imported to the USA at the end of the XIXth century (True 1937, Cottam et al. 1940). Furthermore, Potts (1989) reported that *A. chukar* initially imported into the UK (1966-1967) came from Italy. Overall, these results are in agreement with the homogeneous origin (East Asia) of the only Italian wild chukar population living on Montecristo Island (Barbanera et al. 2007) and of all *A. chukar* genes disclosed in introgressed *Alectoris* birds of the entire Mediterranean (Martinez-Fresno et al. 2008, Barbanera et al. 2009a).

What perspective for the conservation management of *A. rufa rufa*?

Hybridization between *A. rufa* and *A. chukar* is extremely widespread, especially in the easternmost part of the *A. rufa* distribution range, the Italian Peninsula. Barbanera et al. (2010) showed that there is an increasing gradient of *A. chukar* introgression when moving from the western to the eastern side of the *A. rufa* range. Eradication of *A. rufa* x *A. chukar* hybrids in the wild can hardly be effective as such hybridization usually occurs as a full swarm (Allendorf and Luikart 2007). Nevertheless, a more widespread use of the genetic screening of captive-bred birds could be of assistance to contain the spread of chukar genes. Regrettably, as we showed in this paper, the early high conservation value of *A. rufa rufa* introduced to the UK four centuries ago has been quashed by the very recent introduction of another alien species, *A. chukar* and its hybrids with *A. rufa*.

We realize that *A. rufa* is a species of high economic interest especially in the rural areas of the European Union (Blanco-Aguiar et al. 2008). However, a lot of funds and efforts are often devoted to the management of wild *A. rufa* with null conservation value, i.e. of populations of uncertain origin and admixed genetic ID reintroduced for put-and-take hunting (Byers and Burger 1979). Our discovery that the naturalized population of East Anglia is also unsuitable

for the genetic management of native *A. r. rufa* has further reduced the range of options available to carry out concrete conservation action for the populations from the easternmost part of the species' range. Luckily, a study has recently suggested the existence of a genetically well-preserved and self-sustaining *A. rufa* population in the Italian Peninsula (Negri et al. 2013). The phylogenetic placement of this resource (*sensu* Fraser and Bernatchez 2001) within the *A. rufa* puzzle needs further investigation. While, on one hand, we recommend this action be highly prioritized as it represents the most promising attempt for the conservation of the Italian *A. r. rufa*, on the other, we feel that acceptance of triage (*sensu* Wiens et al. 2012) should at least be considered for most of the *A. rufa rufa* populations of Italy.

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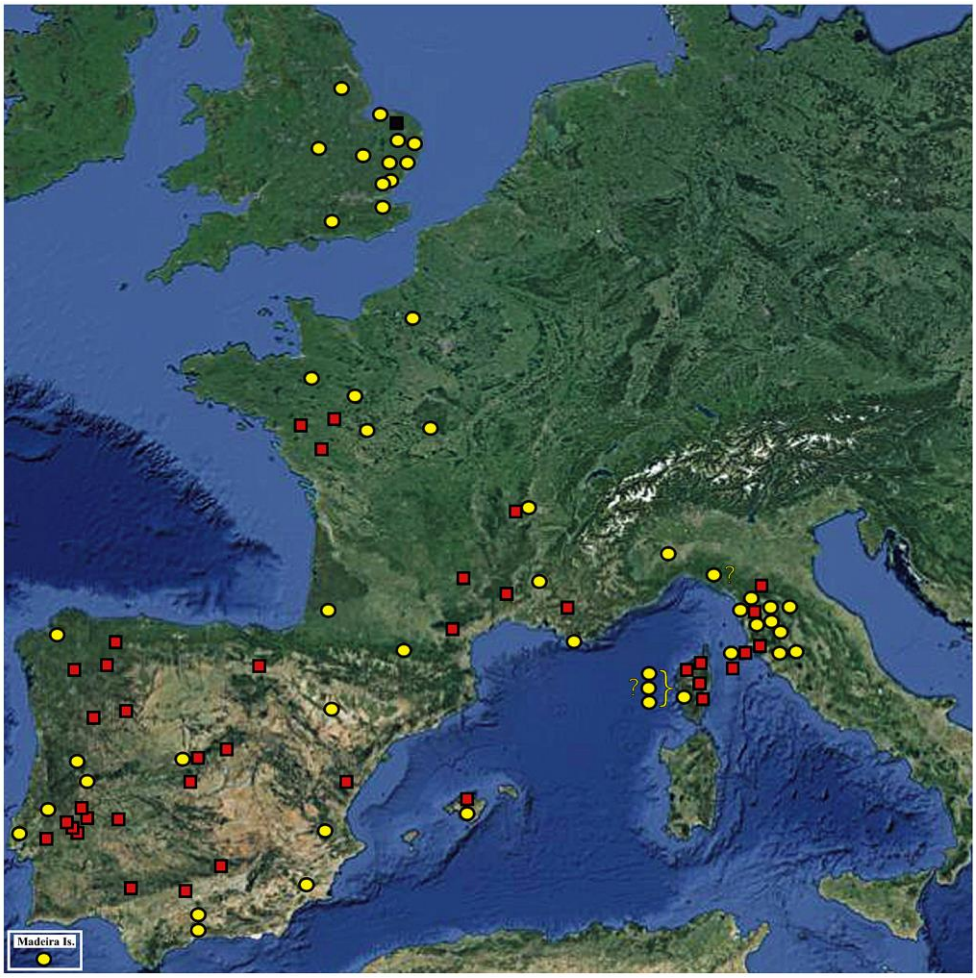
641 **FIGURE AND TABLE LEGENDS**

642 **Fig. 1**. Map showing sampling localities. The single black square refers to the RLP study area
643 in Norfolk (East Anglia); red squares indicate sampled areas across the native range of *A.*
644 *rufa*; yellow circles show where the museum specimens were collected in the wild (Table S1).
645
646 **Fig. 2**. Haplotype (mH1-mH33) network as computed for modern *A. rufa* using 1092 bp -
647 long Cyt-*b* sequences. A scale to infer the number of haplotypes for each pie is provided
648 together with a length bar to compute the number of mutational changes.
649
650 **Fig. 3**. PCA performed using average ϕ_{ST} pairwise distances computed with the *A. rufa* Cyt-*b*
651 sequences (229 bp long) obtained from either modern birds (black circles) or ancient
652 specimens (white circles) held in museums.
653
654 **Fig. 4**. PCA performed using average ϕ_{ST} pairwise distances computed with the *A. chukar*
655 Cyt-*b* + CR (2246 bp) sequences obtained from East Anglian phenotypic RLPs (England:
656 black circle) and chukar partridges from either the native (Greece to China) or introduced
657 (USA) part of the species' range (white circles).
658
659 **Fig. 5**. Bayesian admixture analysis of *A. rufa* (left: 1st parental control), *A. chukar* (right: 2nd
660 parental control) and RLPs from the East Anglian (centre) population genotypes as computed
661 by STRUCTURE with $K = 2$ (white, *A. rufa*; black, *A. chukar*). Each individual is represented as
662 a vertical bar partitioned in K segments, whose length is proportional to the estimated
663 membership in the K clusters.
664

Table 1. The characteristics and the genetic variability of the STR loci for each population: repeated motif; Ta (° C), annealing temperature; size range; *n*, sample size; A_T, number of alleles per locus; A_R, allelic richness; A_P, number of private alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; P_{HWE}, probability value for the Hardy-Weinberg Equilibrium test (Fischer global test, all loci). *, significant departure from HWE after application of the Bonferroni correction ($\alpha = 0.05$, $\alpha' = \alpha/7 = 0.0071$).

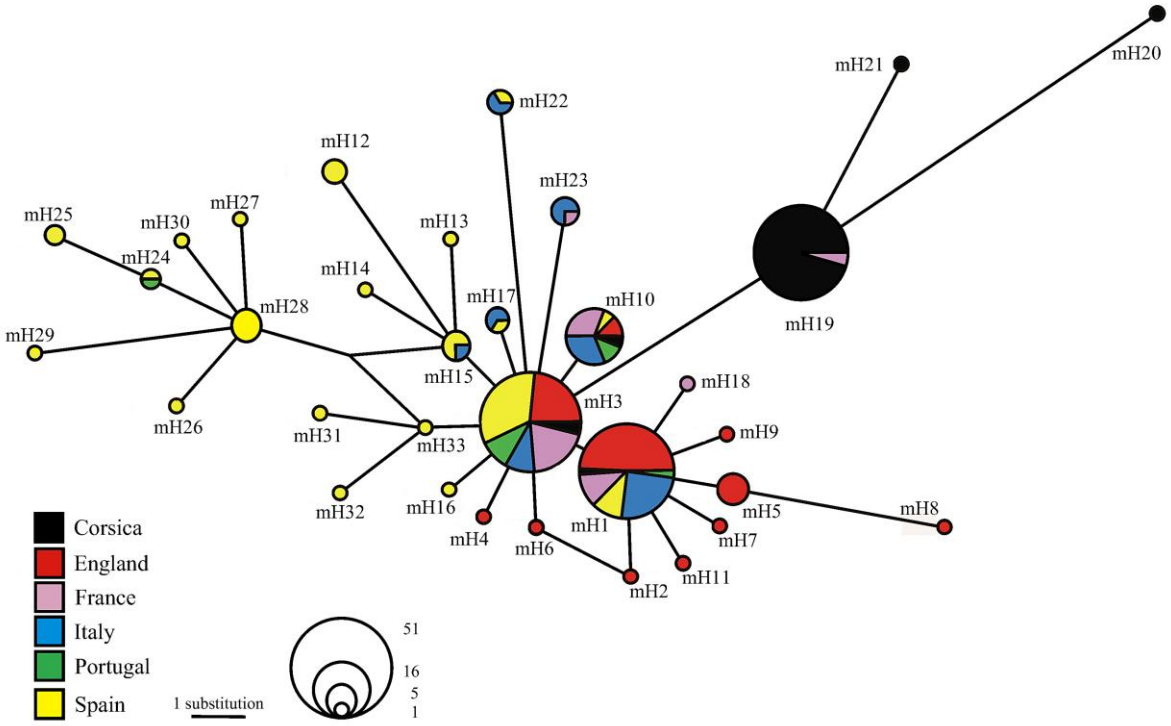
Table 2. The sample size (*n*), the number of haplotypes (N), the number of private haplotypes (N_P) and the haplotype diversity (*h*, with standard deviation) are given for all modern populations (Cyt-*b*: 1092 bp).

Table 3. The sample size (*n*), the number of haplotypes (N), the number of private haplotypes (N_P) and the haplotype diversity (*h*, with standard deviation) are given for all modern and ancient populations (Cyt-*b*: 229 bp). The outcome of the AMOVA (Ap, variability among populations; Wp, variability within populations; all ϕ_{ST} values reported are with $P < 0.001$) is reported either including (values to the left) or not (values to the right) the Corsican *A. rufa*.



689 **Figure 1**

690



691 **Figure 2**

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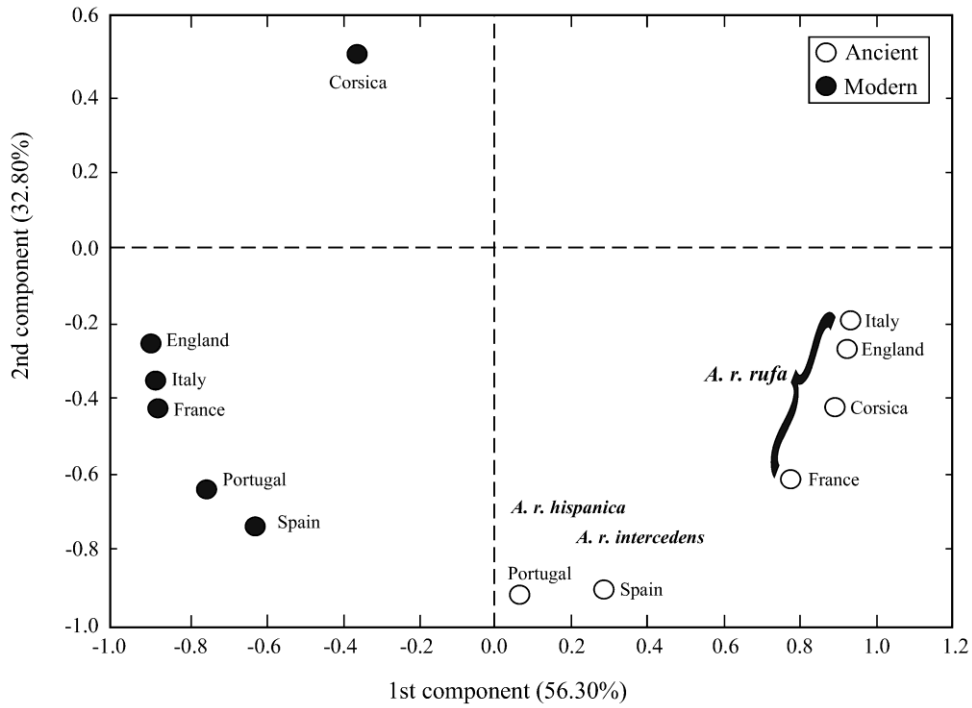
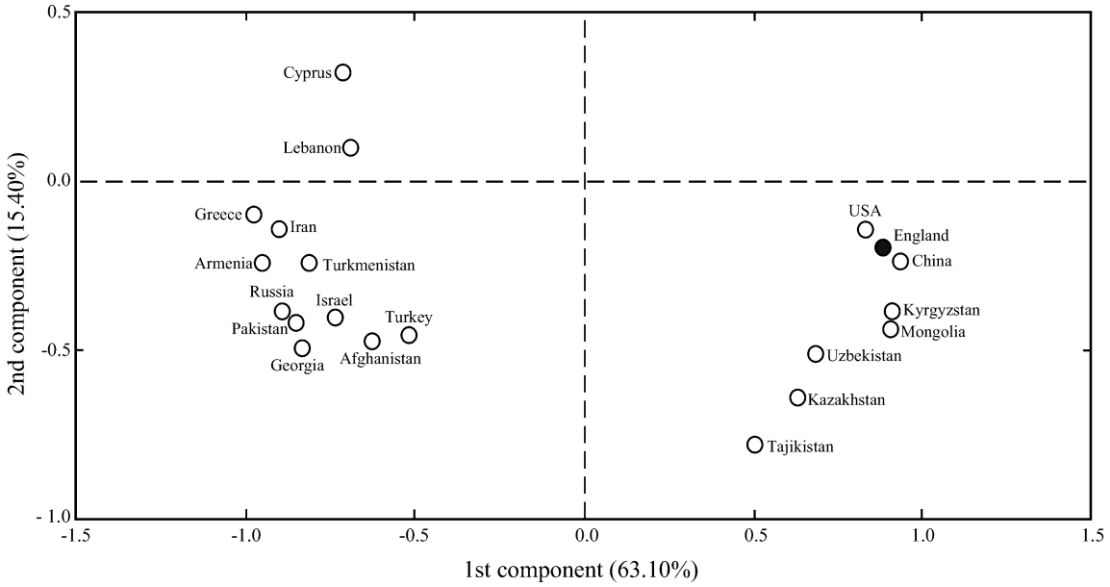

Ancient
Modern
Corsica
England
Italy
France
Portugal
Spain
A. r. rufa
Italy
England
Corsica
France
A. r. hispanica
A. r. intercedens
Portugal
Spain
2nd component (32.80%)
1st component (56.30%)
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2

Figure 3



719 **Figure 4**

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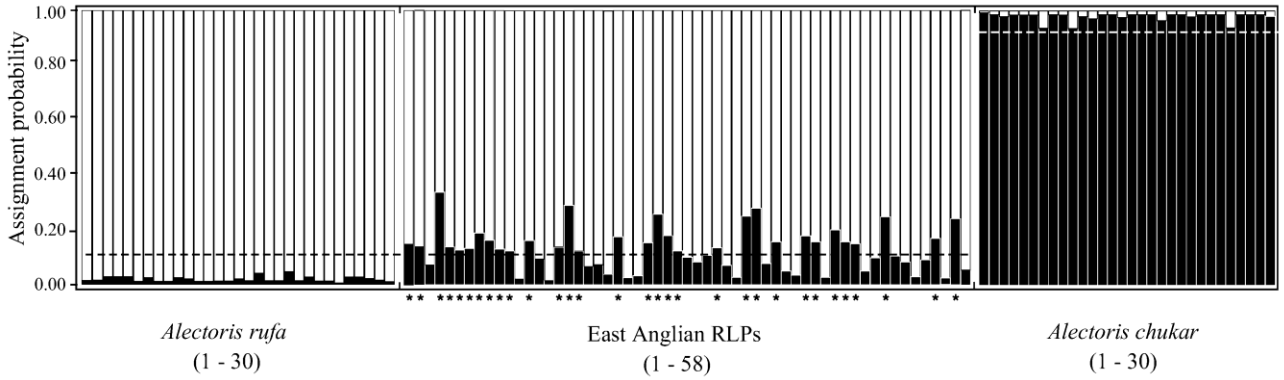
Assignment probability
1.00
0.80
0.60
0.40
0.20
0.00
Alectoris rufa
(1 - 30)
East Anglian RLPs
(1 - 58)
Alectoris chukar
(1 - 30)

Figure 5

				A. rufa						East Anglian RLPs						*A. chukar*					
Locus	Repeated motif	T_a (° C)	Size range (bp)	*n*	A_T	A_R	A_P	H_O/H_E	P_{HWE}	*n*	A_T	A_R	A_P	H_O/H_E	P_{HWE}	*n*	A_T	A_R	A_P	H_O/H_E	P_{HWE}
MCW104	$(TG)_4$	62/55	80 - 126	30	10	9.9	1	0.70/0.88	0.7×10^{-3} *	58	9	7.9	0	0.84/0.85	0.11	30	10	9.9	5	0.67/0.74	0.16
MCW118	$(TA)_2(TATG)_3$ $[TA\ (TATG)_2]_2$ $(TA)_3\ (TATG)$	60/55	143 - 155	29	1	1.0	-	-	-	58	2	1.7	0	0.03/0.03	1.00	30	1	1.0	-	-	-
MCW280	$(AT)_8$	60/55	174 - 180	29	3	3.0	0	0.41/0.41	> 0.99	57	4	3.5	0	0.39/0.56	0.1×10^{-3}*	30	1	1.0	-	-	-
Aru1.23	$(TG)_{13}$	62/55	173 - 187	30	3	3.0	1	0.40/0.43	0.25	58	5	4.3	1	0.29/0.28	0.57	30	3	2.9	0	0.10/0.09	> 0.99
Aru1.27	$(AT)_{13}$	60/55	170 - 200	29	10	10.0	4	0.58/0.56	0.85	58	7	6.8	0	0.67/0.76	0.11	30	6	5.9	0	0.57/0.74	0.01
Aru1E45	$(TG)_9$	60/58	135-173	30	14	13.9	4	0.87/0.87	0.29	58	10	8.4	0	0.91/0.84	0.30	30	8	7.9	3	0.40/0.59	0.01
Aru1I68	$(TG)_{19}$	60/58	225-261	30	6	5.9	1	0.23/0.46	1.7×10^{-3}*	56	8	7.4	2	0.19/0.54	< 0.001*	30	1	1.0	-	-	-
All loci				29.6	46	6.7	11	0.53/0.60	< 0.001*	57.6	45	5.7	3	0.48/0.55	< 0.001*	30	27	4.2	8	0.43/0.54	3×10^{-3}*

Table 1

	Modern *A. rufa* (Cyt-*b*: 1092 bp)			
	n	N	N_P	*h* ± SD
Corsica	48	6	2	0.24 ± 0.08
England	48	11	8	0.73 ± 0.05
France	24	6	1	0.76 ± 0.06
Italy	29	7	0	0.80 ± 0.05
Portugal	10	4	0	0.76 ± 0.13
Spain	48	20	13	0.86 ± 0.04
Total	207	33	24	0.84 ± 0.01

Table 2

	Modern *A. rufa* (Cyt-*b*: 229 bp)				Ancient *A. rufa* (Cyt-*b*: 229 bp)			
	n	N	N_P	*h* ± SD	*n*	N	N_P	*h* ± SD
Corsica	48	4	1	0.20 ± 0.07	4	1	0	0
England	48	4	2	0.20 ± 0.07	13	1	0	0
France	24	4	1	0.52 ± 0.10	14	3	1	0.38 ± 0.15
Italy	29	4	0	0.46 ± 0.10	19	1	0	0
Portugal	10	3	0	0.62 ± 0.14	6	3	1	0.73 ± 0.15
Spain	48	9	4	0.71 ± 0.06	11	3	1	0.62 ± 0.11
Total	207	14	8	0.65 ± 0.03	67	5	3	0.30 ± 0.07
	Amova				Amova			
Ap	45.6/16.6				21.6/22.3			
Wp	54.4/83.4				78.4/77.7			
Φ_{ST}	0.46/0.17				0.22/0.22			

Table 3