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RESEARCH ARTICLE

Genetic diversity and landscape genetic structure of otter (*Lutra lutra*) populations in Europe

Nadia Mucci · Johanna Arrendal · Hermann Ansorge · Michael Bailey · Michaela Bodner · Miguel Delibes · Ainhoa Ferrando · Pascal Fournier · Christine Fournier · José A. Godoy · Petra Hajkova · Silke Hauer · Thrine Moen Heggberget · Dietrich Heidecke · Harri Kirjavainen · Hans-Heinrich Krueger · Kirsti Kvaloy · Lionel Lafontaine · József Lanszki · Charles Lemarchand · Ulla-Maija Liukko · Volker Loeschcke · Gilbert Ludwig · Aksel Bo Madsen · Laurent Mercier · Janis Ozolins · Momir Paunovic · Cino Pertoldi · Ana Piriz · Claudio Prigioni · Margarida Santos-Reis · Teresa Sales Luis · Torsten Stjernberg · Hans Schmid · Franz Suchentrunk · Jens Teubner · Risto Tornberg · Olaf Zinke · Ettore Randi

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Abstract Eurasian otter populations strongly declined and partially disappeared due to global and local causes (habitat destruction, water pollution, human persecution) in parts of their continental range. Conservation strategies, based on reintroduction projects or restoration of dispersal corridors, should rely on sound knowledge of the historical or recent consequences of population genetic structuring. Here we present the results of a survey performed on 616 samples, collected from 19 European countries, genotyped at the mtDNA control-region and 11 autosomal microsatellites. The mtDNA variability was low (nucleotide diversity = 0.0014; average number of pairwise differences = 2.25), suggesting that extant otter mtDNA lineages originated recently. A star-shaped mtDNA network did not allow outlining any phylogeographic inference. Microsatellites were only moderately variable ($H_o = 0.50$; $H_e = 0.58$, on average across populations), the average allele number was low (observed $A_o = 4.9$, range 2.5–6.8; effective $A_e = 2.8$; range 1.6–3.7), suggesting small historical effective population size. Extant

N. Mucci · E. Randi (✉)
Laboratory of Genetics, Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), Via Cà Fornacetta 9, 40064 Ozzano Emilia, Bologna, Italy
e-mail: ettore.randi@infs.it

J. Arrendal
Department of Animal Ecology, Evolutionary Biology Centre, Uppsala University, Norbyv 18D, 752 36 Uppsala, Sweden

H. Ansorge
Senckenberg Museum of Natural History Goerlitz, PF 300154, 02806 Goerlitz, Germany

M. Bailey
Department of Zoology, Trinity College Dublin, College Green, Dublin 2, Ireland

M. Bodner
Stadtplatz 23, 3943 Schrems, Austria

M. Delibes · J. A. Godoy · A. Piriz
Estación Biológica de Doñana, CSIC, Avda Américo Vespucio s/n, 41092 Sevilla, Spain

A. Ferrando
Departament de Biologia Cellular, de Fisiologia i d'Immunologia, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain

P. Fournier · C. Fournier
Groupe de Recherche et d'Etude pour la Gestion de l'Environnement, Route de Préchac, 33730 Villandraut, France

P. Hajkova
Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Kvetna 8, 603 65 Brno, Czech Republic

S. Hauer
Institute of Zoology, Martin Luther University Halle (Saale), Halle (Saale), Germany

T. M. Heggberget
Norwegian Institute for Nature Research, 7485 Trondheim, Norway

D. Heidecke
Institute of Biology/Zoology, Martin-Luther-Universität Halle-Wittenberg, Domplatz 4, 06108 Halle (Saale), Germany

otters likely originated from the expansion of a single refugial population. Bayesian clustering and landscape genetic analyses however indicate that local populations are genetically differentiated, perhaps as consequence of post-glacial demographic fluctuations and recent isolation. These results delineate a framework that should be used for implementing conservation programs in Europe, particularly if they are based on the reintroduction of wild or captive-reproduced otters.

Keywords Eurasian otter · Mitochondrial DNA · Microsatellites · Bayesian clustering · Spatial genetic structure · Landscape genetics

Introduction

The Eurasian otter (*Lutra lutra*) is a top predator living in a variety of aquatic habitats, including rivers, lakes, lagoons, coastal wetlands and marine shores (Kruuk 2006). It is considered a flagship species and an indicator of habitat quality (Bifolchi and Lodè 2005), although otters can breed well also in relatively degraded and less productive streams and wetlands, as long as enough prey is available (Ruiz-Olmo et al. 2001). The species was widely distributed across the Eurasian continent and in parts of North Africa, reaching China, Japan, Indonesia, Malaysia and India (Foster-Turley and Santiapillai 1990). Recently and more markedly during the second half of the last century, many otter populations strongly declined in several European countries, due to a combination of global and local causes. Otters were hunted for fur, or persecuted because they were considered a pest to fish farming and fishery. In some countries a bounty was paid until the 1970s, when the species was finally legally protected. Habitat destruction, such as channeling and mining in or around river beds, dam construction, wetland reclamation and the destruction of riparian forests contributed to eradicate otter populations. Freshwater pollution also destroyed otter populations by killing their prey, or by bioaccumulation of organochlorines and heavy metals (MacDonald and Mason 1994). Now the species is fully protected by the IUCN, CITES and Bern conventions, and by national laws in almost all the European countries.

Strict protection led some otter populations to expand and recover naturally (Kruuk 2006). At the same time, where healthy populations survived, active conservation programs were designed aiming at improving habitat connectivity and sustaining natural dispersal through restored ecological corridors (Reuther 1994). Reintroduction programs of captive-reproduced or relocated wild otters have been planned where natural colonization was no longer possible due to the eradication or isolation of the remaining populations. Reintroduction projects were carried out in southern Sweden (Sjöåsen 1996), Switzerland (Weber et al. 1991), Spain (Saavedra and Sargatal 1998) and the Netherlands (Van Ewijk et al. 1997). Those projects were realized before any information on otter population genetic structure was available (an exception is the recent reintroduction in The Netherlands, from where the otter disappeared after 1989; Koelewijn and Jansman 2007), generating unplanned consequences. Thus, for instance, the

H. Kirjavainen
Department of Biology, University of Joensuu, P.O. Box 111, 80101 Joensuu, Finland

H.-H. Krueger
Aktion Fischotterschutz e. V, Otter-Zentrum, Sudendorfallee 1, 29386 Hankensbuttel, Germany

K. Kvaloy
Norwegian Institute for Nature Research, Tungasletta 2, 7485 Trondheim, Norway

L. Lafontaine
Réseau Loutre Francophone, BP1, 29670 Locquenole, France

J. Lanszki
Department of Nature Conservation, University of Kaposvár, P.O.B. 16, 7401 Kaposvar, Hungary

C. Lemarchand
Ecole Nationale Vétérinaire de Lyon—UMR INRA ENVL 1233, 1, avenue Bourgelat, 69280 Marcy l'Etoile, France

U.-M. Liukko
Finnish Environment Institute, P.O. Box 140, 00251 Helsinki, Finland

V. Loeschcke · C. Pertoldi
Department of Biological Sciences, Ecology and Genetics, Aarhus University, Ny Munkegade, Building 1540, 8000 Aarhus C, Denmark

G. Ludwig
Department of Biological and Environmental Science, University of Jyväskylä, P.O.B. 35, Jyvaskyla 40014, Finland

A. B. Madsen
Department of Wildlife Ecology and Biodiversity, National Environmental Research Institute, University of Aarhus, Kalo, Rønde, Denmark

L. Mercier
Otter Reintroduction Centre, Hunawihr, France

J. Ozolins
State Forest Service, 13 Janvara Iela 15, Riga 1932, Latvia

M. Paunovic
Institute for Biological Research, 29 Novembra 142, Beograd 11000, Serbia and Montenegro

Otter Trust managed a first reintroduction program into lowland English rivers (Wayre 1991), releasing captive-bred otters which showed mitochondrial DNA (mtDNA) haplotypes of non-European origin (Randi et al. 2005). Wild-captured and captive-reproduced otters originating from northern Norway and Sweden were relocated to southern Sweden without any prior knowledge on any possible phylogeographic structure in Scandinavia (Arrendal et al. 2004).

Reintroduction projects should respect the IUCN guidelines prescribing that "the source population of reintroduced animals is genetically as similar as possible with formerly resident genotypes" (IUCN 1998). Thus, information on genetic structure of natural otter populations, as well as the identification of the genetic origins of otters in captivity, should be mandatory before any reintroduction plan is implemented. The intra-specific taxonomy of otter populations is uncertain, because the species exhibits unusually low levels of mtDNA variation, and shows almost no mtDNA geographic structure (Effenberger and Suchentrunk 1999; Mucci et al. 1999; Cassens et al. 2000; Arrendal et al. 2004; Ferrando et al. 2004; Ketmaier and Bernardini 2005; Pérez-Haro et al. 2005; Finnegan and Néill 2009; Stanton et al. 2009). Autosomal microsatellites are polymorphic in otters, but the populations studied so far showed little geographical differentiation also at the nuclear level (Dallas et al. 1999; Pertoldi et al. 2001; Dallas et al. 2002; Randi et al. 2003; Arrendal et al. 2004; Hajkova et al. 2007; Janssens et al. 2008). The scope of published studies was limited by restricted geographical sampling collections. Hence, it is still difficult to evaluate the genetic structure of otter populations in Europe. Two main questions should be answered: (1) do extant natural otter populations show any global phylogeographic differentiation; and (2) did recent anthropogenic demographic fluctuations generate genetic disequilibria and local genetic sub-structuring?

In an effort to answer to these questions, we here present results obtained from a large set of genotypic data, including an alignment of mtDNA sequences (1580 bp long) and multilocus genotypes determined at 11 autosomal microsatellites in 616 otter samples collected from 19 natural populations across the species' distribution in Europe. These data were analyzed using population and landscape genetic approaches aiming at: (1) reconstructing the main patterns of otter genetic differentiation across Europe, and (2) describing detailed otter population structuring at local geographical scale. A broad scale survey across Europe should shed light on eventual phylogeographic structuring of otter populations, and landscape genetic analyses should detect the consequences of recent demographic fluctuations. This information could help in reconstructing the still largely unknown historical biogeography of the species, thus providing guidelines to design sound restoration programs.

Materials and methods

Sample collection

In this study we used a total of 616 distinct otter genotypes. Most of them were determined from 589 tissue samples, preserved in 90% ethanol or Longmire buffer (Longmire et al. 1997) at −20°C, which were collected between 2000 and 2007 from 19 European countries (Table 1). The geographical locations of 535 of these samples (originating from Portugal, Spain, France, Ireland, Germany, Czech Republic, Slovakia, Serbia-Montenegro, Finland, Sweden, Norway and Italy) were mapped using ArcView GIS 3.1 (Fig. 1). The locations of the other 54 samples (collected in England, Ireland, Austria, Denmark, Hungary and Latvia-Belarus) were not precisely known, and thus were not mapped. Additionally, 27 genotypes were obtained from about 200 faecal samples collected in southern Italy (mainly within and around the Pollino National Park; Calabria and Basilicata regions). Otters completely disappeared from north and central Italy before the end of the 1980s, surviving only in the southern regions from where

C. Pertoldi
Mammal Research Institute, Polish Academy of Sciences, Waszkiewicza 1c, 17-230 Bialowieża, Poland

C. Prigioni
Department of Animal Biology, Pavia University, Piazza Botta 9, 27100 Pavia, Italy

M. Santos-Reis · T. S. Luis
Centro de Biologia Ambiental/Departamento de Biologia Animal, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, Ed. C2, 1749-016 Lisbon, Portugal

T. Stjernberg
Finnish Museum of Natural History, Zoological Museum, University of Helsinki-Finland, Helsinki, Finland

H. Schmid · O. Zinke
Zurich Zoo, Zürichbergstrasse 221, 8044 Zurich, Switzerland

F. Suchentrunk
Research Institute of Wildlife Ecology, University of Veterinary Medicine Vienna, Savoyenstrasse 1, 1160 Vienna, Austria

J. Teubner
Landesumweltamt Brandenburg, Naturschutzstation Zippelsförde, 16827 Zippelsforde, Germany

R. Tornberg
Faculty of Science, Department of Biology, University of Oulu, Oulu, Finland

Table 1 Origin and number of the otter (*Lutra lutra*) samples used for mtDNA sequencing and microsatellite (STR) genotyping

Country	Samples	mtDNA	STR
1 Portugal	30	8	30
2 Spain	40	5	40
3 France	42	5	42
4 England	5	1	5
5 Ireland	14	3	14
6 Denmark	15	5	15
7 Germany	170	18	170
8 Austria	18	13	18
9 Czech Republic	27	–	27
10 Slovakia	15	2	15
11 Hungary	6	4	6
12–13 Serbia and Montenegro	8	–	8
14–15 Latvia and Belarus	6	1	6
16 Finland	74	17	74
17 Sweden	43	7	43
18 Norway	69	3	69
19 Italy	34	3	34

Samples from Serbia and Montenegro ($n = 8$), and from Latvia and Belarus ($n = 6$) were pooled

the samples used in this study were obtained (Prigioni et al. 2006).

Tissue samples derived mainly from found-dead or trapped otters collected from regions where the species is more abundant. Therefore, sampling was not homogenous across Europe, but most of the more widespread populations are represented. Natural otter populations are expanding in Spain and Portugal (Ruiz-Olmo et al. 2001). Our samples derive mainly from central and south-western regions (Extremadura, Andalucia, Faro, Beja, Setubal, Evora, Lisboa, Santarem, Portalegre) where the species is particularly abundant. Only a few individuals were collected from northern Spain. We did not get samples from central and eastern Spain where otters are rare or absent. French samples derived from the Atlantic west coast provinces (Bretagne, Pays de la Loire, Poitou–Charentes, Aquitaine). Only a few samples were collected from the Central Massif. Otters recently expanded westwards throughout most of eastern Germany (Reuther and Roy 2001) and we obtained a large number of samples from the whole range (Saxony, Brandenburg and Mecklenburg). Sampling was done fairly evenly within the distribution of the species in Czech Republic, Slovakia, Finland, Sweden and Norway. We collected samples also from southern

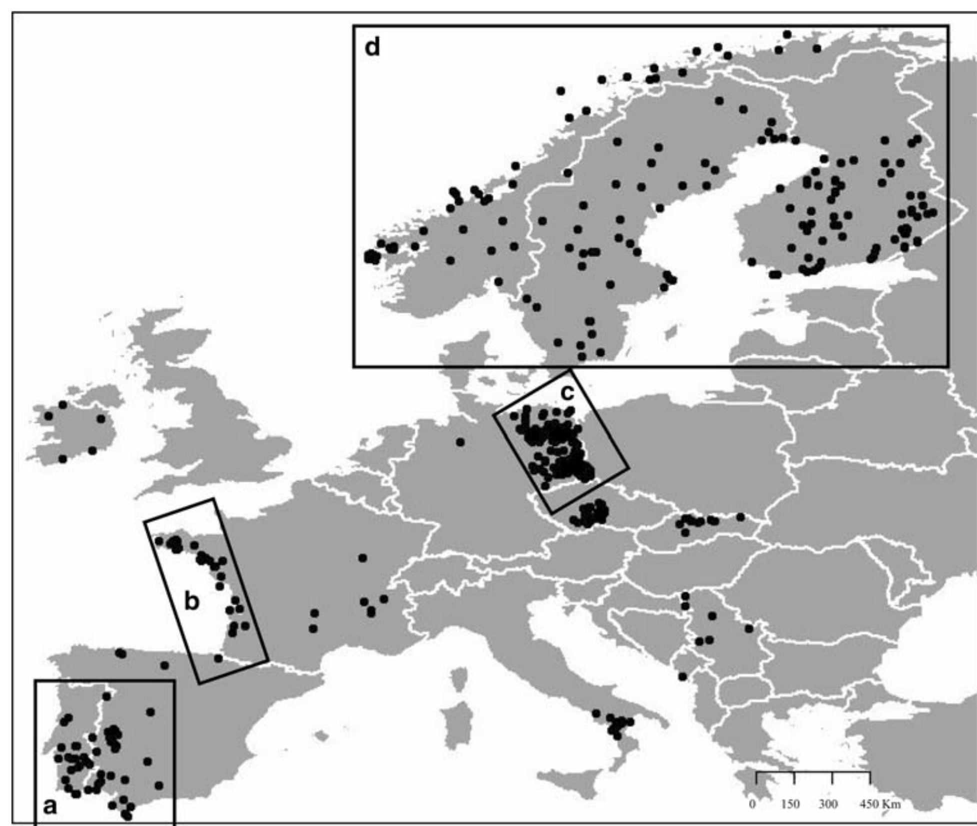

Fig. 1 Origin of the otter samples in Europe. Four individuals from Ireland and the samples collected in England, Austria, Denmark, Hungary and Latvia-Belarus are not mapped because their geographical locations were not available. *Rectangles* identify those populations which were analyzed separately in GENELAND (see: Results and Fig. 5)

Sweden where otters were reintroduced in the past 20 years (Sjöåsen 1996). We did not use additional samples from East Anglia (UK) because those populations originated from the reintroduction of otters by the Otter Trust breeding centre (Jefferies et al. 1986). Otters are widespread in north-west Austria where they are in contact with the Czech population. A disjunct expanding otter population is distributed in south-east Austria (Reuther 1994). Our samples originated from both populations, although no detailed geographic information was made available. The Danish population is restricted to northern and western Jutland (Madsen 1996) from where the samples were collected. Only a few individuals were available from Serbia and Montenegro, Latvia, Belarus and England.

DNA extraction, amplification and genotyping

Total DNA was extracted using a guanidine-thiocyanate and silica beads protocol (Gerloff et al. 1995). DNA tied to silica particles was cleaned using sequential washings and finally eluted in a TE buffer (10 mM TrisHCl, pH 8; 0.1 mM EDTA). Most of the published mtDNA studies are based on partial sequences of the control-region, which in otters show unusually low sequence variation. In order to search for additional mutations in other mtDNA regions, we sequenced ca. 2000 bp, including the 3′ end of the cytocrome *b* (CYB; 65 bp), the threonine tRNA (tRNA-Thr; 68 bp), the proline tRNA(tRNA-Pro; 66 bp), the entire control-region (CR; 1090 bp), the phenilalanine tRNA (tRNA-Phe; 69 bp) and the initial 5′ region of the 12 ribosomal RNA gene (12S RNA; 464 bp), which was PCR-amplified in 95 samples, selected to represent all the sampling locations, using the external primers *LlucybL996* (5′-CCT TAC CCT AAC CTG AAT CGG) and *12SH51* (5′-CTA GAG GGA TGT AAA GCA CCG). Amplifications were performed in a 9700 ABI thermal cycler using the following protocol: (94°C × 2′), 40 cycles at (94°C × 40″) (50°C × 40″) (72°C × 1′), and a final extension at 72°C for 10′. Clean sequences of ca. 1822 bp were obtained directly from the PCR products, with the PCR primers, the forward primers *LLU-dL225* (5′-CCC AAG ACT CAA GGA AGA GGC), *OTT-D3L* (5′-ACA ACA TTT ACT GTG CCT GCC C), *OTT-D4L* (5′-CAT CTG GTT CTT ACT TCA GGG CC), and the reverse primer *OTT-D5H* (5′-ACA AGT GGT GGG AGA GAG AAG CG) using an ABI 3130XL automated sequencer. Sequences were analyzed using Sequencing Analysis 5.3 and SeqScape 2.5 (Applied Biosystems). A final alignment of 1580 bp was obtained using Bioedit 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) after the removal of a variable length repeated region (242 bp long). Additionally, a shorter fragment 479 bp long, including the final 3′ end of the CYB (65 bp), tRNA-Thr (68 bp), tRNA-Pro (66 bp) and the initial part of the CR (280 bp), was sequenced in 15 faecal individual genotypes from southern Italy using primers *Llucy*bL996 and *H16498* (5′-CCTGAACTAGGAACCA-GATG-3′).

Multilocus genotypes of 616 samples were obtained by PCR amplifications of the following 11 autosomal microsatellites: Lut435, Lut453, Lut604, Lut701, Lut715, Lut733, Lut782, Lut818, Lut832, Lut833 (Dallas and Piertney 1998) and Lut902 (Dallas et al. 1999). Amplifications were performed using the following protocol: (94°C × 2′), 35 cycles at (94°C × 30″) (60°C × 30″) (72°C × 1′), and a final extension at 72°C for 10′. Alleles and genotypes were identified using an ABI 3130XL sequencer and the software GeneMapper 4.0 (Applied Biosystems).

Analyses of the mtDNA sequences

The sequences were aligned with a mtDNA sequence of the European otter (GenBank NC_011358), and the haplotypes were identified using Collapse 1.0 (http://crandalllab.byu.edu/Computer.aspx). Haplotype diversity (h), average pairwise nucleotide substitutions (k), and other statistics were computed using Dnasp 5.00.07 (Rozas et al. 2003). Unrooted networks were drawn to infer haplotype relationships with the median-joining network procedure as implemented in Network 4.5.1.0 (Bandelt et al. 1999; http://www.fluxus-engineering.com/sharenet.htm). The distribution of observed pairwise haplotype substitutions (mismatch distribution) was computed with Arlequin 3.1.1 (Excoffier et al. 2005; http://cmpg.unibe.ch/software/arlequin3/), and a population-expansion test was performed using the sum of square deviation (SSD) between the observed and the expected mismatch, and the Harpending's raggedness index (R; Schneider and Excoffier 1999). Tajima's D (Tajima 1989) was computed using the segregating sites method in Arlequin.

Analyses of microsatellite variation

Population genetic analyses were performed in two ways. First, we analyzed pre-defined groups corresponding to the sampled countries, which could, admittedly, include a number of genetically distinct, but unknown, biological populations. The few samples from Serbia and Montenegro, and from Latvia and Belarus were aggregated in population genetic analyses. The software GenAlEx 6.1 (Peakall and Smouse 2006) was used to compute, for each of the pre-defined groups, the observed and effective average number of alleles per locus (A_o and A_e) and the average expected and observed heterozygosity (H_e and H_o). The software Genetix 4.03 (Belkhir et al. 2001) was used to test for departure from Hardy–Weinberg equilibrium (HWE) through the values of the fixation index F_{IS} (Wright

1969; the probability to obtain simulated F_{IS} values higher than the observed was evaluated after 1,000 random permutations of alleles within individuals) and Factorial Correspondence Analyses (FCA; Benzecri 1973) plotting individual multilocus genotypes in 2- or 3-D Cartesian spaces. A Principal Component Analysis (PCA) of differentiation among the sampled populations was performed with PCA-GEN 1.2. (http://www2.unil.ch/popgen/softwares/pcagen.htm).

Second, we used untrained Bayesian clustering (with software STRUCTURE; Pritchard et al. 2000) to split the sampling groups into a number of sub-populations that could correspond to natural genetically distinct groups (see details below). The geographic structure of the subpopulations was further investigated through landscape genetic analyses (with the software GENELAND 3.1.5; http://www2.imm.dtu.dk/~gigu/Geneland/#; Guillot et al. 2005). The genetic structure of pre-defined and new sub-populations was described using: (1) the number of microsatellite loci in which a departure from HWE was observed; (2) the fixation index F_{IS}; (3) the average F_{ST} values among sub-populations; (4) an estimation of isolation-by-distance (IBD) through a Mantel test of correlation between genetic and geographic distance matrices (latitude and longitude were used to assess the geographical distance among individuals); and (5) the Paetkau et al. (2004) population assignment test.

Bayesian inference of population structure

The genetic structure of the sampled populations was inferred using the multilocus genotypes and the Bayesian clustering procedures implemented in STRUCTURE 2.2.1 (http://pritch.bsd.uchicago.edu/structure.html). STRUCTURE was designed to identify the number K of distinct genetic populations (clusters) included in the sample, assuming HWE and linkage equilibrium within each population, and to assign the individuals to the inferred clusters. Burn-in periods of 50,000 steps followed by 500,000 Monte Carlo iterations were used to obtain convergence of the parameter values. Explorative analyses were performed first with K from 1 to 18 using all the samples, then splitting the sample into six distinct geographical subgroups (see below). All simulations were independently replicated four times for each K, using the “admixture” and the “independent” allele frequency models (Falush et al. 2003). The number of populations K was set at the value that maximised the increase in the posterior probability of the data LnP(D) according to the formula $[\mathrm{LnP(D)}_k - \mathrm{LnP(D)}_{k-1}]$ (Garnier et al. 2004). Individual samples were assigned to the clusters using only genetic information, and ignoring sampling locations (options *usepopinfo* = 0, *popflag* = 1). For each K, the coefficients of individuals membership q_i were used to assign probabilistically the genotypes to one cluster (the population of origin), or to more than one cluster if they were admixed. Averaged coefficients of membership across the four replicates were obtained by CLUMPP 1.0 (Jakobsson and Rosenberg 2007; http://rosenberglab.bioinformatics.med.umich.edu/clumpp.html). The software DISTRUCT 1.1 (Rosenberg 2004; http://rosenberglab.bioinformatics.med.umich.edu/distruct.html) was used to plot the graphical representations of the q_i values.

Landscape genetic analyses

Based on the results of the first STRUCTURE analyses, and taking into account the geographical locations of the sampled populations, we identified six main subgroups (506 samples; see Results), which were analysed with GENELAND to determine simultaneously the population genetic structure and the geographical distributions of the clusters. This procedure uses information on genotypes and geographical locations to infer the spatial structure of the samples, assuming that spatial proximity should be *a-priori* related to genetic proximity. Genetic and geographic distances among individual genotypes were used to maximize the posterior probability to obtain the optimal number of clusters K and their spatial locations. The density distribution of the number of populations after a burn-in period of 50,000 iterations was used to select the optimal K value. Then, five replicates of 100,000 MCMC iterations (with thinning = 1,000) using the “independent” allele frequency model were run. At optimal K values, the posterior mode of population membership was used to assign the individuals to the subpopulations. The geographical distributions of the subpopulations were reconstructed from the plottings of the posterior probability of each individual to have origins in each of the K clusters.

Results

The mtDNA network

The long mtDNA alignment (95 individuals, 1,580 nucleotides) showed 20 distinct haplotypes, defined by 20 polymorphic sites (19 transitions and one indel, including 15 singletons and only four parsimony informative sites). No mutations were detected in the CYB, tRNA-Thr and tRNA-Phe genes. One diagnostic transition and one indel characterized the tRNA-Pro of the Italian samples. There were five transitions (0.8% sequence divergence), one transversion and three transitions (1.5% sequence divergence), respectively in the first (593 bp) and in the final part (255 bp) of the CR. Finally, nine transitions (1.9% sequence divergence) were found in the 12S rRNA gene (465 bp). Mitochondrial DNA diversity was high, with

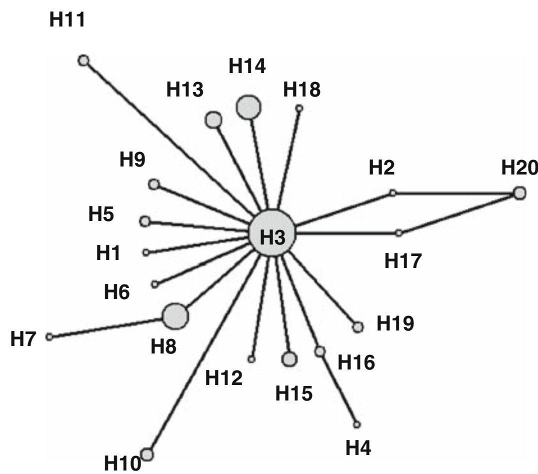

Fig. 2 Minimum spanning network connecting the otter mtDNA haplotypes included in this study ($n = 95$). Each *circle* represents a single haplotype and the area of the *circle* is proportional to its frequency. The length of the lines are proportional to the number of mutations (short segments = one mutation; long segments = two mutations). The central haplotype (H3) is widespread in all Europe (except in southern Italy). Other haplotypes, differing in one or two mutations respect to the H3 haplotype, are distributed in the European countries. H1, H2 (Denmark); H5 (Germany, Hungary); H17 (Hungary); H19, H20 (Austria); H8, H9, H12, H18 (Germany); H7 (Ireland); H4 (UK); H13 (Spain and Portugal); H10 (Italy); H11 (Finland, Norway); H6, H16 (Sweden); H14, H15 (Finland)

one distinct haplotype every 4.75 individuals. Overall haplotype diversity was high (0.79 ± 0.037, SD), but nucleotide diversity (0.0014 ± 0.00012) and average number of pairwise differences (2.25) were low, suggesting that extant otter mtDNA lineages originated recently. The network was star-shaped and did not show any obvious signal of phylogeographic structuring (Fig. 2), with a central haplotype (H3) that is widespread in all European countries, with the exception of populations from southern Italy, which showed only haplotype H10 (Table 2). The additional shorter sequences (from 15 faecal samples), confirmed that only H10 is present in the southern Italian otters. The widespread haplotype H3 corresponds to H1 and Lut1, as reported respectively by Ferrando et al. (2004) and Stanton et al. (2009). Haplotypes H8 and H14 were found only in Germany and in Finland with frequencies of 67 and 59%, respectively. Most of the haplotypes differed from H3 by one mutation (Fig. 2). The unimodal mismatch distribution indicated a sudden demographic expansion at *Tau* = 2.5 (90% confidence interval = 1.1–3.7). The raggedness index was $R = 0.382$; the probabilities to obtain by simulations SSD and R values > than the observed were = 0.001. Tajima's D = −2.187 was significantly negative ($P < 0.01$), consistent with a signal of demographic expansion. Assuming a star-shaped genealogy and that the

Table 2 Distribution of the otter mtDNA haplotypes in the sampled countries: *P* Portugal, *SP* Spain, *FR* France, *UK* United Kingdom, *IR* Ireland, *DK* Denmark, *D* Germany, *A* Austria, *SK* Slovakia, *HU* Hungary, *B* Belarus, *FIN* Finland, *SW* Sweden, *NW* Norway, *IT* Italy

Haplotype	P	SP	FR	UK	IR	DK	D	A	SK	HU	B	FIN	SW	NW	IT	Total
H1						1										1
H2						1										1
H3	5	3	5		2	3	1	8	2	2	1	2	4	2		40
H4				1												1
H5							1			1						2
H6													1			1
H7					1											1
H8							12									12
H9							2									2
H10															3	3
H11												1		1		2
H12							1									1
H13	3	2														5
H14												10				10
H15												4				4
H16													2			2
H17										1						1
H18							1									1
H19								2								2
H20								3								3
Total	8	5	5	1	3	5	18	13	2	4	1	17	7	3	3	95

otter mtDNA evolved at the "standard" mammalian mtDNA rate of ca. 2% sequence divergence/million years (Avise 1986), the estimated average number of pairwise differences (2.5/1580 bp) could have been generated in ca. 100,000–150,000 years of evolution from a common ancestral mtDNA genome. This date is congruent with a simple phylogeographic model assuming that all extant European otter populations derive from the postglacial expansion of a single small population that survived isolated in a refuge area during the last glaciation.

Microsatellite diversity

Estimates of genetic diversity obtained from the analysis of 11 microsatellites in 616 otter samples are shown in Table 3. All loci were polymorphic in all sampled populations. The average allele number per locus was $A_o = 4.9$, and ranged from four (Czech Republic) to seven (Norway) alleles, with the exception of populations sampled in southern Italy and Denmark, which showed only 2.5 and 2.9 alleles per locus, respectively. The average effective allele number per locus was $A_e = 2.8$, ranging from the lowest values in Denmark and Italy ($A_e = 1.6$) to the highest value in Sweden ($A_e = 3.7$). Observed and expected heterozygosity was moderate ($H_o = 0.50$; $H_e = 0.58$), on average across populations. Observed heterozygosity was lowest in samples from Denmark and Italy ($H_o = 0.35$ and 0.37, respectively), and highest in some northern populations (i.e. otters from Latvia, Belarus, Finland and Sweden, showing $H_o > 0.65$). Values of H_e were always greater than values of H_o, with the exception of England, Serbia-Montenegro, and Latvia-Belarus where $H_e < H_o$, and Slovakia and Italy where H_o and H_e did not differ. Thus, the F_{IS} values were significantly greater than zero in 10 of 17 locations, indicating widespread significant departures from HWE in those sampling groups, while samples from isolated populations (i.e. southern Italy, Slovakia, Czech Republic, Ireland) were in HWE (Table 3). Therefore, otters from the sampled geographical locations showed moderate levels of microsatellite allelic diversity, which was particularly low in some isolated groups, such as otters in Denmark and southern Italy. Moreover, most of the sampling groups assembled on the base of their country of origin, were not in HWE, showing less than expected heterozygotes, which possibly indicates a Wahlund effect (Wahlund 1928).

Table 3 Genetic diversity in sampling groups as estimated at 11 microsatellite loci

Population	n	A_o	A_e	H_e	H_o	F_{IS}	P-value	
Portugal	30	4.7	2.8	0.60	0.56	0.09	0.009	**
Spain	40	5.8	3.2	0.64	0.58	0.11	<0.001	**
France	42	4.8	2.6	0.59	0.48	0.20	<0.001	**
England	5	2.5	1.9	0.43	0.46	0.04	0.344	n.s.
Ireland	14	4.2	2.7	0.59	0.58	0.06	0.122	n.s.
Denmark	15	2.9	1.7	0.39	0.35	0.14	0.024	*
Germany	170	6.3	3.4	0.65	0.58	0.11	<0.001	**
Austria	18	4.6	2.5	0.57	0.44	0.25	<0.001	**
Czech Republic	27	3.9	2.3	0.51	0.50	0.04	0.187	n.s.
Slovakia	15	4.4	3.0	0.58	0.58	0.03	0.262	n.s.
Hungary	6	3.9	3.0	0.64	0.53	0.26	<0.001	**
Serbia-Montenegro	8	4.4	3.2	0.66	0.67	0.06	0.160	n.s.
Latvia-Belarus	6	4.3	3.0	0.63	0.69	0.00	0.427	n.s.
Finland	74	6.6	3.6	0.70	0.65	0.08	<0.001	**
Sweden	43	6.6	3.7	0.71	0.65	0.09	<0.001	**
Norway	69	6.8	3.3	0.65	0.50	0.24	<0.001	**
Italy	34	2.6	1.7	0.37	0.37	0.02	0.360	n.s.

n sample size, A_o average number of alleles per locus, A_e average number of effective alleles per locus, H_e expected heterozygosity, H_o observed heterozygosity, F_{IS} fixation index; significance of the F_{IS} probability value (P-value); *n.s.* not significant; * $P < 0.05$, ** $P < 0.05$

Global genetic population structure

The FCA plot of multilocus genotypes showed limited geographical differentiation among most of the sampled otter populations (Fig. 3a). The FCA did not provide evidence of clearly distinct clusters, although the individuals sampled from the various populations were not evenly distributed in the multivariate space, but showed a sharp tendency to clump according to their geographical origins. Along the first FCA component (that described 3.76% of the total variance) all Iberian otters were located towards the upper right corner of the plot, while most of the otters sampled from Germany were located towards the upper left corner. Along the second component (explaining 2.72% of the total variance) a group of the samples from Norway (the southern Norwegian samples) were distributed separately in the lower region of the FCA (Fig. 3a). Along the third component (explaining 2.70% of the total variance) all the Italian otters clustered in the lower region of the plot, separately from all the other samples (Fig. 3b). The PCA performed with PCA-GEN confirmed these results, showing that populations from Iberia, Italy and Germany clustered separately from all the others (Fig. 3c). In this analysis there were two significant principal components, which explained 26.69% (PC I) and 19.50% (PC II) of the total inertia. This means that the populations separated along the first and second PCA components respectively showed $F_{ST} = 0.034$ and $F_{ST} = 0.024$, corresponding to 27 and 19% of the total F_{ST} value of 0.126. The same results were obtained by an FCA on populations computed using GENETIX (not shown).

An analysis of molecular variance (AMOVA), calculated in GENALEX, showed that 17% of the total genetic

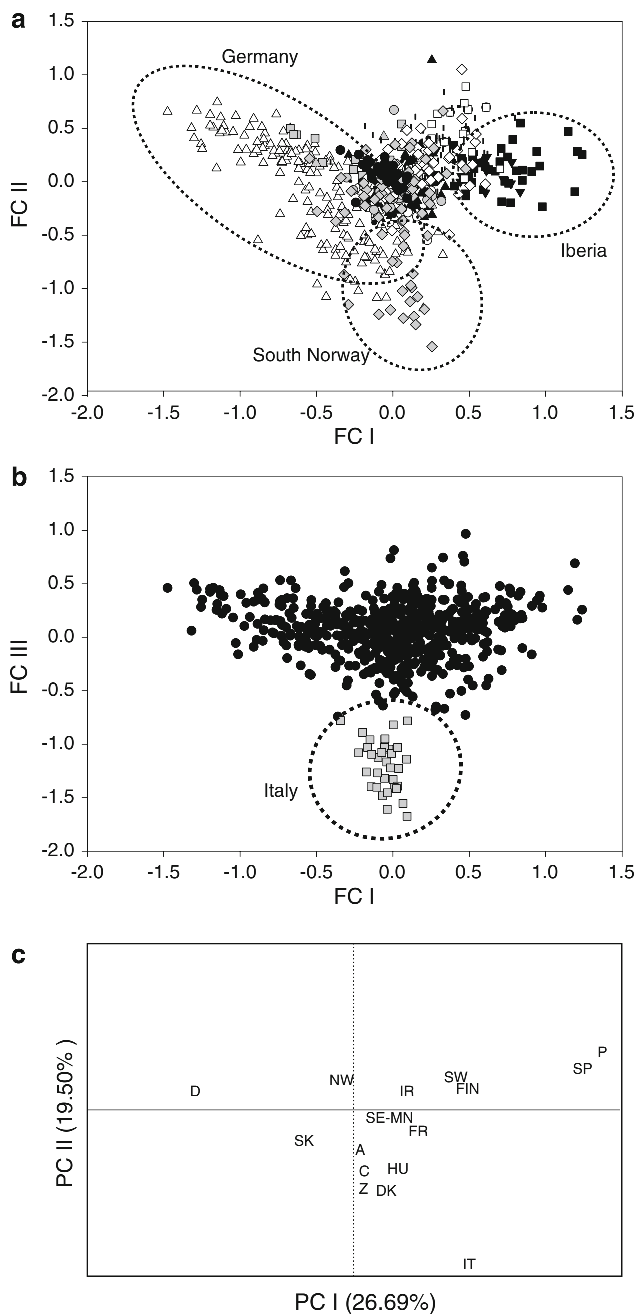

Fig. 3 Factorial correspondence analysis (FCA) of otter individual (**a** and **b**), and principal component analysis (PCA) of otter population (**c**) multilocus scores computed using GENETIX and PCA-GEN (**c**)

variability was significantly distributed among countries ($P = 0.001$). This geographic partition of genetic variability generated significant global values of $F_{ST} = 0.13$ (95% confidence interval = 0.10–0.16, computed after 1000 bootstraps over loci), and $F_{IS} = 0.12$ (95% confidence interval = 0.11–0.13). In 136 comparisons among the 17 sampling locations, there were only six non-significant pairwise F_{ST} values ($P > 0.05$), which were due to comparisons between otters sampled in: (1) Austria and Czech Republic, Hungary, Latvia-Bielorussia; and (2) Hungary and Serbia-Montenegro and Latvia-Bielorussia.

Population sub-structuring

STRUCTURE analyses, performed using all the samples and no prior information on their locations, showed likelihood values that slowly tended to reach a plateau at $K > 10$ (Fig. 4a), while showing the maximum ΔK increases between the initial values, that is, from $K = 2$ to 8. Increasing the K values led to define the number of distinct clusters, and to clarify the admixture patterns within clusters. To summarize the complex sub-structuring and admixture patterns, we show the averaged q_i plotting (population admixture proportions; Fig. 4b, upper), and the individual admixture proportions (Fig. 4b, lower), in the clusters as defined at $K = 11$. The south Italian otter population was clearly identified and showed no admixture (right end of the plot). The two Iberian populations (Portugal and Spain) joined into a single cluster that showed some admixture signals, particularly among the Spanish individuals (left end of the plot). Other local populations, which were consistently sampled, showed variable aggregation and admixture patterns: otters from France and Germany showed evidence of sub-structuring and admixture, while otters from Fennoscandia showed strong signals of individual admixture. Otters from Austria and Czech Republic joined the same cluster, which was distinct from the Slovakian and other eastern European otter samples. Isolated (and nor well sampled) otter populations from UK and Denmark also joined distinct clusters.

Most of these patterns were already evident at lower K values (Fig. 4c). At $K = 3$ all Iberian otters clustered together, showing little admixture. At $K = 3$ otters from Germany already showed a sharp splitting into two distinct groups, a result that was also confirmed at higher K values. Genetic heterogeneity among Fennoscandian otters was mainly due to evidences for two distinct subpopulations in otters sampled from Norway, which was already clear at $K = 3$. The individual admixture patterns showed that most of the populations included a number of admixed individuals, with the exceptions of Italy and Denmark. All the other populations showed a number of admixed individuals, mainly in France, Slovakia and Fennoscandia. Some individuals sampled from Germany and Norway were also strongly admixed. These findings highlight evidences of genetic distinctions of otters sampled form the southern extremes (southern Iberia and Italy) of the species' distributions, and call for more detailed analyses that can be performed using landscape genetic approaches.

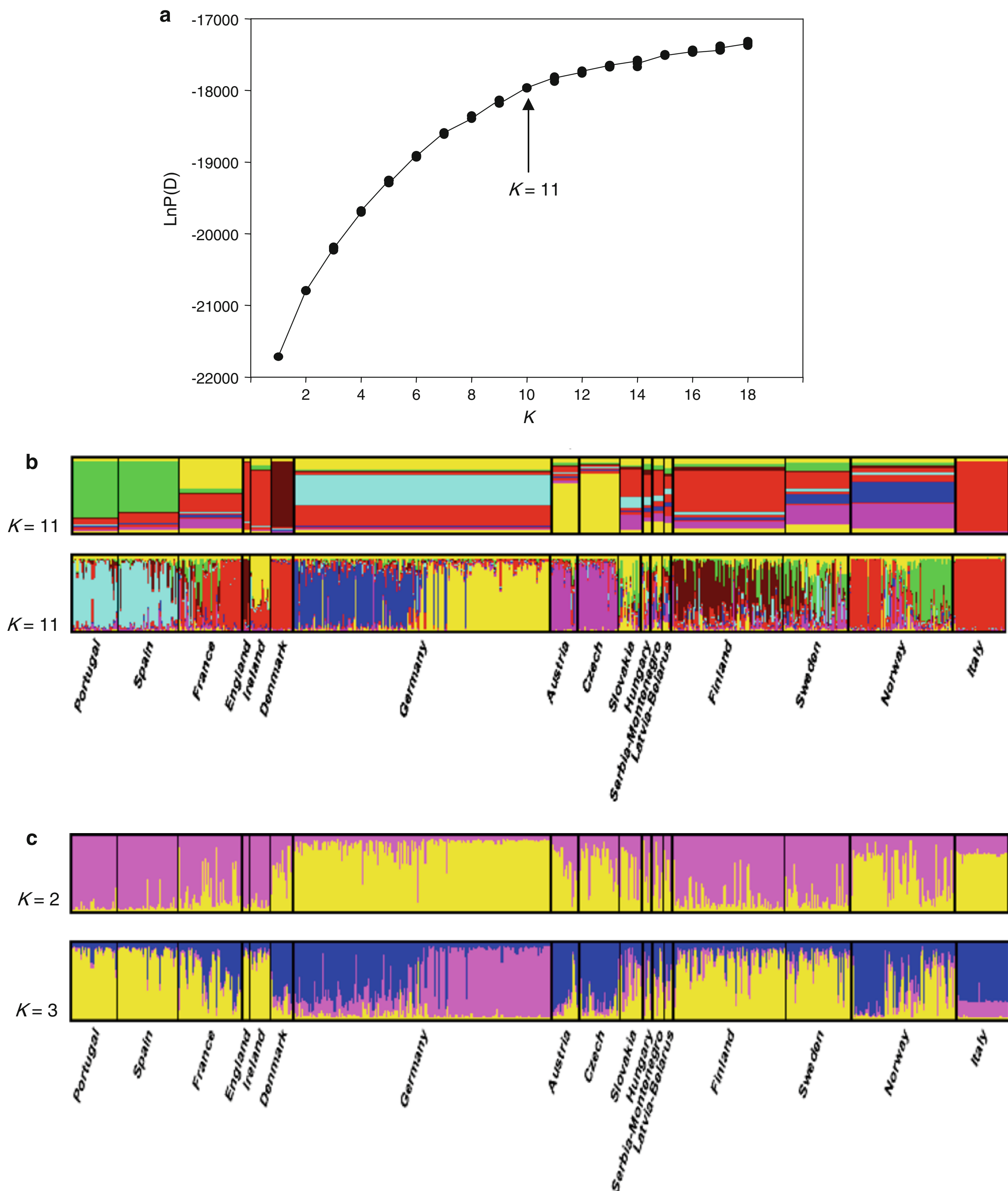

Fig. 4 Estimated population structure in otter. **a** Mean log-likelihood values for four replicated STRUCTURE runs with prior K values ranging from 1 to 18. **b** Population clustering (*upper*) and individual admixtures (*lower*) plottings for $K = 11$. **c** Individual admixtures (*lower*) plottings for $K = 2$ and 3. Each individual is represented as a vertical bar partitioned into one or more segments; the length of segments being proportional to the individual membership values (q_i)

Landscape genetics of otter populations

The existence of large geographical gaps among population distributions and sampling locations, prevented us to perform a global pan-European landscape genetic analysis. Therefore, we used the main clusters from the STRUCTURE results and geographical proximity of sampling locations to identify six groups of otter samples: (1) Iberia (central-southern Portugal and Spain; excluding 4 samples from northern Spain); (2) France (only samples collected along the Atlantic coast; seven isolated samples from north-east France and Massif Central were excluded); (3) central Europe (including otters from Germany, Slovakia, Czech Republic, Serbia and Montenegro); (4) Germany; (5) Slovakia and Czech Republic; (6) Fennoscandia (Norway, Sweden and Finland). These groups were further analyzed using STRUCTURE and GENELAND aiming to infer their spatial genetic structure. Isolated populations (England, Ireland, Denmark, Italy), poorly represented geographical groups (Hungary, Latvia, Belarus) and populations lacking of geographical information (Austria) were excluded from the following analyses. Results showed that:

1) Iberia. Otters sampled from Iberia were split into two sub-populations by both GENELAND (Fig. 5a) and STRUCTURE (not shown). The sub-population defined by cluster 1 included the otters sampled from Coimbra in the centre of Portugal, and from Extremadura, Castilla-La Mancha and León in central Spain. Cluster 2 included the otters sampled from Santarém, South Portalegre, Lisbon, Evora, Setubal, Beja, Faro in south Portugal, and from South Extremadura and Andalusia in south Spain. As it is shown in Table 4, this splitting is supported by: (a) smaller deviations from HWE (lower F_{IS} values in the sub-populations than in the pooled Iberian population); (b) highly significant F_{ST} value between sub-populations 1 and 2; (c) strongly reduced isolation-by-distance (IBD) in sub-population 1 as assessed through the Mantel test; and d) 91% individuals that were correctly assigned to the sub-populations 1 or 2 from which they were originally sampled.
2) France. Samples collected in France were split into three sub-populations (Fig. 5b), although an alternative splitting into four sub-populations was also partially supported by both GENELAND and STRUCTURE (not shown). The small sample size ($n = 6$) of sub-population 3 prevented us to estimate reliably the optimal K and the value of the population genetic parameters. Deviation from HWE was significantly reduced only in sub-population 1, which did not show any detectable IBD effect and no mis-assigned individuals (Table 4). The average F_{ST} was highly significant.

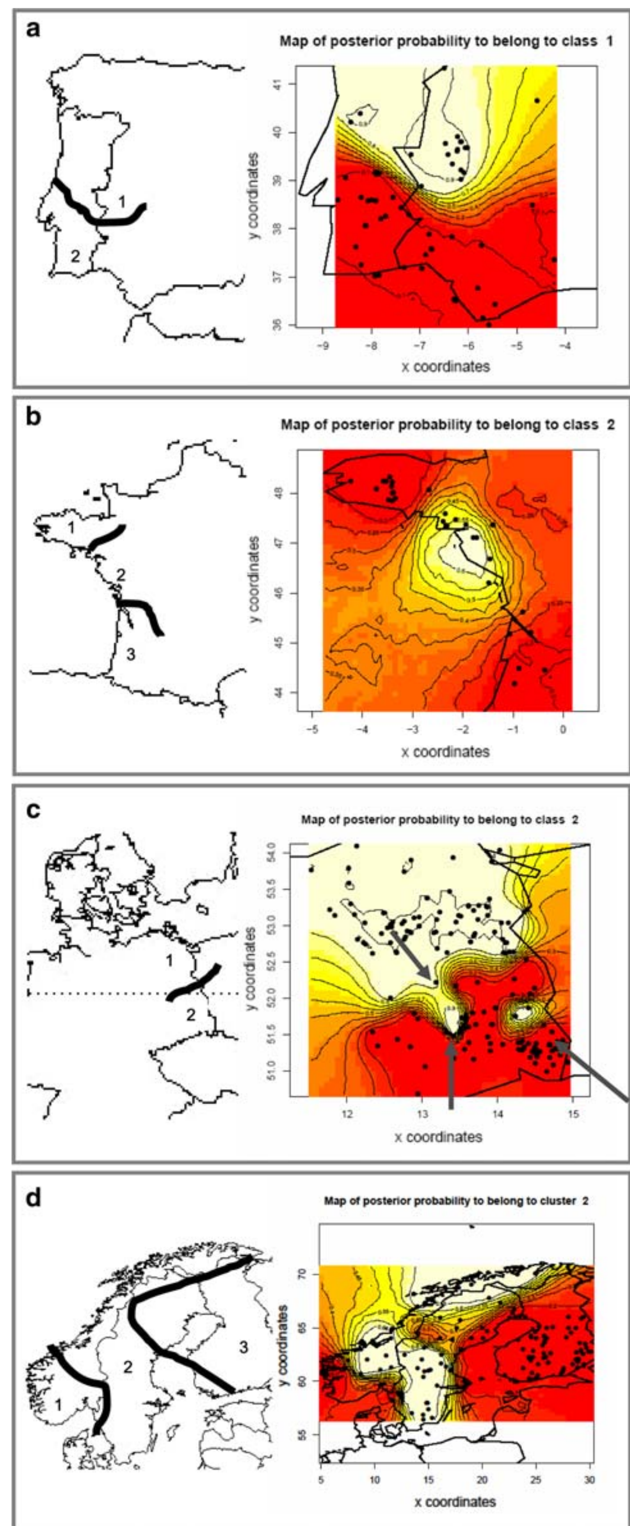

Fig. 5 Maps of the individual posterior probability to belong to distinct genetic clusters as identified by GENELAND in the following geographic areas: **a** Iberian Peninsula; **b** Western France; **c** Germany; **d** Fennoscandia (see also Fig. 1). The highest membership values of individuals are identified by white areas

Table 4 Subdivision of sampling groups according to Bayesian analyses performed using STRUCTURE

Population	K	Subpop	HWE	F_{ST}	P	F_{IS}	P	R_{xy}	P	Assign
Iberia	2	Tot (70)	2	0.116	0.000			0.22	0.000	91%
		1 (48)	0	0.085	0.004			0.13	0.027	2 (2)
		2 (18)	1	0.086	0.036	0.057	0.000	0.38	0.017	4 (1)
France	3	Tot (35)	1	0.188	0.000			0.44	0.000	89%
		1 (15)	1	0.166	0.006			0.16	0.052	0
		2 (12)	0	0.000	0.600			0.54	0.000	1 (1) 1 (3)
		3 (6)	0	0.106	0.014	0.155	0.000	nc	nc	2 (1)
Central Europe	4	Tot (219)	5 + 2	0.131	0.000			0.20	0.000	91%
		1 (79)	2	0.058	0.007			0.05	0.212	4 (3)
		2 (22)	0	0.075	0.033			0.41	0.000	2 (3) 1 (4)
		3 (90)	1	0.067	0.000			0.19	0.000	5 (1) 6 (2)
		4 (28)	2	0.066	0.041	0.101	0.000	0.12	0.117	1 (2)
Germany	2	Tot (165)	6	0.110	0.000			0.37	0.001	97%
		1 (75)	0	0.035	0.058			0.01	0.417	0
		2 (90)	1	0.075	0.000	0.105	0.000	0.18	0.001	5 (1)
Slovakia–Czech Republic	2	Tot (42)	2 + 2	0.118	0.000			0.46	0.001	100%
		1 (27)	2	0.040	0.148			0.19	0.030	0
		2 (15)	2	0.035	0.238	0.163	0.000	0.17	0.022	0
Fennoscandia	3	Tot (186)	7 + 2	0.172	0.000			0.26	0.000	94%
		1	4	0.160	0.000			0.20	0.000	4 (3)
		2 (2 sub)	0	0.123	0.007			0.49	0.002	0
		3	2	0.082	0.000	0.099	0.000	0.07	0.098	7 (1)

K number of sub-populations identified by STRUCTURE, *Subpop* individuals for each population or subpopulation, *HWE* number of loci that showed a departure from HWE, F_{IS} fixation index and its significance (P), F_{ST} average F_{ST} values among sub-populations and its significance (P), R_{XY} Mantel test of correlation between genetic and geographic distance matrices and its significance (P), *Assign* Assignment test (Paetkau et al. 2004) as implemented in GENEALEX

3) Otters from central Europe (Germany, Czech Republic, Slovakia, Serbia and Montenegro) are fragmented in a number of genetically distinct clusters. Both GENELAND and STRUCTURE (data not shown) indicate an optimal value of $K = 4$. This splitting reduced the deviations from HWE both in terms of loci that were not in equilibrium in the pooled population vs. the four sub-populations, and in terms of F_{IS} values. However, significant signals of IBD persisted in sub-populations 2 (corresponding to otters from Slovakia, Serbia and Montenegro) and 4 (otters from Czech Republic). Sub-populations 1 (Germany) and 3 were also not in HWE, and showed a number of mis-assigned individuals (Table 4). These results invited us to further investigate the sub-population structure at lower geographical scales in Germany, Czech Republic and Slovakia.
4) Samples collected in Germany were split into two sub-populations by STRUCTURE and GENELAND (Fig. 5c). Sub-population 2 included otters from south Brandenburg and Saxony, while sub-population 1 grouped samples from upper Brandenburg and Mecklenburg, except for four probably mislabeled individuals (indicated by the arrows in Fig. 5c). The splitting into two subpopulations strongly reduced the number of loci not in HWE and the F_{IS} values, particularly in population 1 that also showed a reduction of IDB. The F_{ST} between sub-populations 1 and 2 was significant (Table 4).
5) Otters from Czech Republic and Slovakia were split into two sub-populations by GENELAND and STRUCTURE (data not shown), according to their geographical origins. The two sub-populations were in HWE and there were no signals of IBD; 100% of the samples were correctly assigned (Table 4).
6) The sub-structure of the Fennoscandian samples was not clearly resolved with this data set: three sub-populations were detected by both STRUCTURE and GENELAND (Fig. 5e), although an alternative splitting into four populations was also supported by STRUCTURE (not shown). Sub-population 1 included only samples collected in south-west Norway; sub-population 2 otters collected in north and central Norway and central Sweden; sub-population 3 grouped samples from Finland and north Sweden. The pooled samples

were strongly deviating from HWE, and IBD was very significant (Table 4). However, also the three sub-populations were not in HWE; the IDB was reduced in the sub-populations, with the exception of population 2. Nevertheless, 94% of the individuals were correctly assigned. Interestingly, the sub-population located in south-west Norway was assigned to a distinct cluster, suggesting genetic isolation.

Discussion

Lack of phylogeographic structure and origin of the European otter populations

The otter is one of the few mammalian species that, despite being globally abundant and widely distributed throughout the Eurasian continents, displays very low diversity at the mtDNA CR, and no apparent phylogeographic structure (Randi et al. 2005). Results from previous studies, obtained by partial (300 bp) or complete (1,100 bp) sequencing of the mtDNA CR, showed that a single haplotype is predominantly distributed in almost all the otter populations sampled across Europe, from the Iberian Peninsula to Scandinavia and Russia. Most of the other haplotypes are restricted to single or few localities and the major part of the sampled localities showed just one or a few haplotypes. In this study we obtained extended sequences, including the 3′ end of the CYB, the entire CR and the initial 5′ region of the 12S RNA, which confirm the published results. The vast majority of otter mtDNA haplotypes differ by 1–2 mutations across more than 1,500 nucleotides, and most of the population groups host just a few haplotypes. The mtDNA sequences showed a star-shaped phylogeny that makes the identification of putative sources of colonization events impossible, and that cannot be used to describe any phylogeographic pattern. The otter populations are not reciprocally monophyletic and hence it is not possible to use mtDNA information to design Evolutionary Significant Units (ESU, *sensu* Moritz 1994), with the exception of the population from southern Italy, which, congruent also with the microsatellite results, might represent an ESU.

The CYB gene (Koepfli and Wayne 1998) and complete mitochondrial genome sequences (Ki et al. 2009) were used in phylogenetic studies of the subfamily Lutrinae, and showed that otters speciated recently. Koepfli et al. (2008) sequenced the CYB and NADH5 genes (1,832 bp in total) in otters from Eurasian localities, showing again that a single very frequent haplotype was widespread, and that most of the haplotypes found in Europe differed just by one or a few mutations. However, three haplotypes sampled from South Korea were more divergent (1.15% uncorrected distance) and joined into a distinct phylogeographic clade (bootstrap support from maximum-likelihood trees = 100%). Although a recent selective sweep cannot be excluded, these findings suggest that extant European otters originated recently, and that western Europe was colonized by the expansion of a single refugial population. The observed average sequence divergence (0.16% uncorrected distance) could have been generated in ca. 100,000–150,000 years of evolution from a common ancestral mtDNA genome. This result fits well with recent assessments of the European sub-fossil record. The only known Pleistocene otter bones were discovered in northern Italy (Fiore et al. 2004), dated early Weichselian (the Wuerm glaciation). All the other 473 known otter sub-fossil remains were collected only in Holocene deposits (Sommer and Benecke 2004; Sommer and Nadachowski 2006). Otters became frequent and geographically widespread only from 5500 BC, and reached north Europe and the British islands only around 3000 BC. Otter remains are absent from both western and eastern Europe since Mid Holocene (during the Atlantic and Sub Boreal periods, 5500–3000 BC). Hence, sub-fossil and mtDNA data consistently indicate that otters colonized central Europe during the Holocene, most probably from a single refugial population that survived in isolation during the entire last glaciation. Although neither the sub-fossil record nor the mtDNA data can be used to locate the refuge area, it has been hypothesized that otters survived only in the Italian Peninsula (Sommer and Nadachowski 2006), although eastern refugia have also been indicated (Ferrando et al. 2004).

Landscape genetic structure of otter populations

Microsatellite allelic diversity and heterozygosity were moderate in the European otter populations, concordant with other published studies (Dallas et al. 1999, 2002; Pertoldi et al. 2001; Arrendal et al. 2004; Hajkova et al. 2007). In this study, we could not find evidence for any clear global trend in the geographical distribution of genetic diversity. Otters in central Europe (Germany) and Fennoscandia showed the highest numbers of alleles per locus and average heterozygosity. In contrast, other north European populations (e.g. Denmark) showed the lowest genetic diversity values, probably due to post-Pleistocene bottlenecks (Pertoldi et al. 2001; Randi et al. 2005). Consequently, the microsatellite data did not indicate any clear phylogeographic patterns. For instance, genetic diversity does not decline northwards as it is expected from serial bottlenecks during colonization waves, or it does not increase in central European regions as expected by

post-glacial admixtures of expanding differentiated populations (Hewitt 2000). These scenarios are not supported perhaps because the historical phylogeographic patterns have been disrupted by the consequences of more recent climate change or anthropogenic population declines and fragmentation. Randi et al. (2003) using Beaumont's (1999) MSVAR procedure (http://www.rubic.rdg.ac.uk/~mab/software.html) suggested that otter populations in Europe suffered two strong demographic declines ca. 4700–4900 and 2000-2600 years ago, respectively. A sudden otter population decline in Denmark, probably caused by human disturbance ca. 2000–3000 years ago, was described by Pertoldi et al. (2001). Hajkova et al. (2007) detected signals of a recent decline (during the last century) that affected the genetic composition of otter populations in the Czech and Slovak republics.

In summary, genetic data indicates that the Holocene history of European otters has been dominated by a global expansion wave followed by local demographic fluctuations, which left detectable signals in the genetic make-up of the populations. Most of the groups, corresponding to the sampled countries, that we used in the global population genetic analyses were not in mutation-drift equilibrium, showing significant departure from HWE and suggesting that they do not represent random breeding populations, but rather artificial admixture of populations which are at least partially isolated (Wahlund 1928). These artificial groups were split, through Bayesian clustering and landscape genetic analyses, in a number of clusters that represent more natural sub-populations. In this way, otters distributed in Iberia were split into two sub-populations, genetically distinct, which are currently in contact in regions (Coimbra and Portalegre) that are not characterized by obvious physical or habitat barriers. Otters distributed along the western Atlantic French coast and in eastern Germany were sampled through apparently continuous ranges, which however included respectively three and two cryptic sub-populations. We could not identify any obvious extrinsic barrier separating these sub-populations. Otters from Fennoscandia were also subdivided into at least three sub-populations, not delimited by any obvious geographical barrier. Most of the inferred sub-groups were more close to HWE and showed less IBD than their corresponding geographical aggregations. The assignment tests also showed that almost all the individuals could be assigned to their respective sub-population of origin. Thus, local otter populations in Europe are genetically subdivided, and their cryptic structure is discovered using multilocus markers and landscape genetics methods.

Genetic sub-structure might be maintained by restricted contemporary gene flow (Dallas et al. 2002). Significant patterns of IBD are evident at the widest geographical scales covered by this study (e.g. ca. 600 km in Iberia, 600 km in France, 400 km in Germany, 2000 km in Fennoscandia), but they are partially reduced at the smaller geographical scales defined by the cryptic sub-group subdivisions (ranging from 140 to 1600 km; ca. 260 and 410 km in the populations 1 and 2 of Iberia; ca.120, 200 and 180 km in populations 1, 2 and 3 of France; ca. 200 and 200 km in the populations 1 and 2 of Germany; ca. 140, 1600 and 1600 km in the populations 1, 2 and 3 of Fennoscandia). This findings mean that local populations mate randomly and are connected by gene flow at distances not wider than a few hundred km, in agreement with results of Dallas et al. (2002). Despite the potential for high dispersal (Durbin 1996), intrinsic factors, such as natal philopatry and polygyny, or extrinsic habitat barriers (e.g. topography and watershed structure, regions of unsuitable habitats) can hamper gene flow and dispersal. Alternatively, those local populations which are currently expanding after recent declines and fragmentation events, like the otter populations in Germany, might have had no time enough to admix and reach stable genetic equilibria.

Conclusions: population genetics and otter conservation

This study generalizes at a continental scale early findings showing that European otters do not present any obvious phylogeographic pattern, but that extant local populations are isolated by distance and deviate from HWE. Concordantly with results described by Dallas et al. (2002) in British otters, also the populations sampled in south Iberia, central Europe and Fennoscandia showed cryptic substructuring, which could have not been predicted simply by their spatial distributions. A continent-wide lack of phylogeographic structure did not prevent the onset of fine-grained population sub-structuring. Consequently, otters in Europe are currently subdivided in a mosaic of sub-populations generated by both historical (fragmentation) and current (limited dispersal) factors.

Conservation strategies, either if based on habitat restoration or animal translocations, should take into account these information. Habitat restoration programs are aimed at facilitating the expansion of extant natural populations. However, evidences of IBD, detectable at a scale of a few hundred km, and limited gene flow indicate that effective otter dispersal distances are spatially restricted. It follows that source populations should not be geographically too distant from putative colonization areas (Dallas et al. 2002). Many otter distribution areas remain to be better sampled, and detailed landscape genetic analyses need to be performed case-by-case, to identify those critical landscape features that can limit

otter dispersal within and between river basins (Janssens et al. 2008).

In areas in which natural colonization is not possible or natural connections are not available, reintroduction programs are considered a viable alternative. In Europe reintroduction programs were based on the release of wild-captured or captive-reproduced otters. In these cases, preliminary assessments of the genetic structure of the founders, are mandatory, in order to avoid the release: (1) of animals that originated from crossings between European and Asian otters (Wayre 1991), which are known to bear mtDNA haplotypes of non-European origin (Mucci et al. 1999; Randi et al. 2003; Ferrando et al. 2004); (2) of highly inbred otters with low genetic variability. The dynamics of reintroduced populations should be carefully monitored, also using non-invasive genetic methods. The outcome of reintroduction projects can vary if evaluated at local or more widespread geographic scales. Released otters might survive and reproduce successfully locally, but the genetic structure of more distant population might be not affected (Arrendal et al. 2004).

Both habitat restoration and animal translocations could lead to admixture of populations that have been historically isolated and genetically differentiated. Admixture would increase genetic diversity and reduce inbreeding, or, in contrast, results in a loss of local adaptation and increases the risk of outbreeding depression (Edmands 2007), depending on the past demographic history of the admixing populations. Local adaptations could originate rapidly also in large and mobile carnivores and generate genetically differentiated ecotypes (Musiani et al. 2007). The adaptive consequences of otter isolation in different habitats types are, at the moment, unknown. More detailed landscape genetic analyses and monitoring of ongoing translocation projects, could eventually lead to identify populations that are better suited to survive, for instances, in poor versus rich food resource areas, or in coastal versus inland ecosystems. For these reason, waiting for better identification of eventual genotype-habitat covariance, we suggest cautions in planning translocation of otters among very different habitat types.

Finally, results in this study offer, for the first time at a continental scale, a collection of allele frequencies that can be used as a guideline to design non-invasive genetic projects, suited to monitor the dynamics of reintroduced populations, including apparent survival, sex ratios, dispersal and effective gene flow (Jansman et al. 2001; Arrendal et al. 2004; Hajkova et al. 2007). In particular, this data set identifies baseline populations that can be used to detect the presence of contemporary migration from neighboring populations, or the presence of alien genotypes that could derive from the release of captive bred otters of non-European origins.

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