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BIODIVERSITY RESEARCH

Multiple founder effects are followed by range expansion and admixture during the invasion process of the raccoon (*Procyon lotor*) in Europe

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ABSTRACT

Aim Understanding colonization dynamics is crucial for management of invasive species. We compare the genetic structure of historical (Central Europe) and recent (Spain) invasive populations with native and captive populations of the North American raccoon (*Procyon lotor*). Our aim was to analyse the effects of colonization age on genetic population structure, understand the role of captive individuals as potential founders and test the role of rivers for the dispersal of the species.

Location North America, Spain, Central Europe.

Methods We genotyped wild-caught raccoons from Spain and Central Europe ($N = 596$), zoos ($N = 57$) and the native range ($N = 153$) at 16 microsatellite loci and sequenced a mitochondrial DNA fragment (Control Region). We analysed population genetic structuring with Bayesian assignment methods and a FCA. In a landscape genetic analysis, we tested the effect of waterways in the dispersal of the species.

Results We detected 16 genetic clusters (in BAPS), supporting the hypothesis of multiple introductions and ongoing releases in the invasive range. The native population showed nearly no genetic structure, the Central European clusters showed signals of admixture, whereas the Spanish clusters were clearly separated. Admixture of the Central European clusters was probably caused by recent contact of populations with different origin. The landscape genetic analysis showed that rivers represent neither barriers nor corridors in Central Europe.

Main conclusions As the Spanish populations are genetically more diverse than the Central European, we expect increased within-population diversity when the still isolated populations merge after range expansion. As our results provide evidence for gene flow between zoos and free-ranging populations, better control of pet trade is essential in the management efforts concerning this invasive species. Our study shows that genetic analyses can help to reconstruct invasion processes, which is important for better understanding and effective management of invasive species.

Keywords
invasion genetics, invasion pathways, invasive species, landscape genetic study, microsatellite, range expansion, wildlife management.

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INTRODUCTION

Invasions of non-native species are of major concern as they can threaten native biodiversity and may affect the economy (Kettunen *et al.*, 2009). Despite the knowledge on the negative effects of invasions, there is a continuous increase on invasions world-wide due to deliberate or accidental releases of organisms as well as international trade and transport (Hulme *et al.*, 2008). Although there is increasing information concerning patterns and processes of invasions, the genetic consequences of invasions are still insufficiently understood. Critical stages of the invasion process after the introduction are the initial establishment of a population and the spread in the new region (Lockwood *et al.*, 2013). Multiple introductions of individuals of distinct sources and releases of a large number of individuals (high propagule pressure) can help to overcome genetic founder effects and may increase the genetic diversity of a population and therefore their probability of survival, their establishment and subsequent spread (Sakai *et al.*, 2001; Dlugosch & Parker, 2008; Schulte *et al.*, 2012). Genetic admixture after secondary contact of several non-native populations can facilitate their adaptability and thereby accelerate range expansion (Kolbe *et al.*, 2004; Keller & Taylor, 2010). For effective management of invasive species, it is important to detect such processes, to identify introduction pathways and factors determining the genetic structure in the new range that may contribute to a successful invasion.

The raccoon (*Procyon lotor*) is native to North America, but invasive in parts of Europe and Asia (DAISIE, 2016). In the past, the raccoon was commercially farmed and bred for its fur, while currently it is kept in zoos and wildlife parks but also by private owners as pet. Besides escapees also deliberate introductions were made for hunting and environmental enhancement in Germany during the last century creating viable populations (Beltrán-Beck *et al.*, 2012). Contrary to the historical records, which assumed that the invasion in Europe stems from two introduction events of a small number of individuals in 1934 and 1945 in Germany (Hohmann & Bartussek, 2011), recent genetic studies have shown that there were at least four independent introductions to Germany which were done in the mid of the last century but also in the mid-1990s (Frantz *et al.*, 2013; Fischer *et al.*, 2015). In other European countries, the raccoon was introduced more recently. Besides the main route of entry from Germany to other European countries, which has been shown for the Polish and Czech raccoon populations (Biedrzycka *et al.*, 2014), private keeping of animals is a further source of non-native populations. First records in Spain were reported in 2001, most likely originated from the pet trade (García *et al.*, 2012). A genetic study on raccoons from Central Spain recognized two independent introduction events with a small effective founder size (Alda *et al.*, 2013). The authors also showed that the two subpopulations may come into contact near Madrid, which may increase their invasive capability. Furthermore, records from other parts of the country exist, indicating that there is a continuous introduction of individuals (García *et al.*, 2012).

The existence of historical and recent invasive populations in Central Europe and Spain provides us with the unique opportunity, to study differences in the genetic structure and genetic diversity of these, which may help to understand the temporal development of genetic structuring in invasive populations. As multiple and secondary introductions, especially of individuals from genetically disparate source populations, may increase invasion success (Schulte *et al.*, 2012), tracing pathways of gene flow is of major importance for the management of invasions. We therefore studied the population genetics of invasive, native and captive populations of the raccoon. As a former study of the Central European raccoon population has shown that the number of introductions was larger than expected (Fischer *et al.*, 2015), it is crucial to understand whether escapees from captive populations still contribute to the wild population. We thus (1) studied the mitochondrial haplotypes of the control region to test the hypothesis that new records of the raccoon at the invasive range margin stem from independent introductions rather than from range expansions. As the number of haplotypes in the native range is quite large (76 haplotypes; Cullingham *et al.*, 2008), whereas it is low in Central Europe (six haplotypes, Frantz *et al.*, 2013), the discovery of haplotypes that have not been recorded so far from Central Europe would indicate a new introduction. Furthermore, we (2) genotyped individuals from the Central European and Spanish invasive range, the native North American range as well as from zoo populations to compare the genetic structure of these raccoon populations. We also used these data to test the hypothesis that (3) the more recent population (Spain) is genetically more similar to the source population in North America than the historical population in Central Europe and (4) to test whether gene flow between zoo and wild populations still occurs. During dispersal, linear features of the landscape (e.g. rivers, hedges) are important for animal movement as they may act as corridors or barriers to movement (Van Der Windt & Swart, 2008). For raccoons, rivers may be of particular importance for dispersal (Cullingham *et al.*, 2009). We therefore also tested (5) whether rivers influence the genetic structure of the invasive population using a landscape genetics approach.

METHODS

Sampling and DNA extraction

A total of 806 raccoon samples (508 tissue, 153 precipitated DNA, 119 hair, 26 buccal swab samples) was collected in Central Europe (Germany, Belgium and Luxembourg), Spain (Madrid, Castilla-La Mancha, Andalusia, Galicia, Catalonia, Balearic Islands and Valencia) and from 12 states and

provinces in North America (Alabama, Florida, Kansas, Maine, New Brunswick, New York, Ohio, Ontario, Pennsylvania, Quebec, Tennessee and Vermont). A subset (193 samples and 407 samples, respectively) of the total of 495 samples collected from Central Europe were also included in previous studies (Frantz *et al.*, 2013; Fischer *et al.*, 2015). Samples were collected from trapped, road-killed or legally hunted individuals in the wild as well as from 57 individuals kept in zoos or wildlife parks in Germany. Buccal swabbing was performed with sterile diagnostic rayon dry swabs (Copan Diagnostics Inc., Murrieta, CA, USA), which were stored at −20 °C. Tissue samples were stored frozen or in absolute ethanol. Hair samples were stored on filter paper with silica gel (ThoMar OHG, Lütau, Germany) at room temperature. DNA samples from North America were precipitated and shipped dry and dissolved in Milli-Q water at the destination. Genomic DNA from tissue or swab samples were extracted using the DNeasy Blood and Tissue kit (QIAGEN, Hilden, Germany), following the manufacturer's protocol (replacing ATL buffer with 200 μL PBS buffer for the swab samples). We extracted the hair DNA with a modified Chelex 100 protocol, using 250 μL of a 10% Chelex 100 Resin solution (Bio-Rad, München, Germany) with addition of 4 μL proteinase K (18 mg mL^{-1}) per sample and an overnight lysis step (Walsh *et al.*, 1991).

Mitochondrial DNA sequencing

We amplified a fragment of the mitochondrial control region (535–587 bp) using the forward primer *PLO-L15997*; 5′-CCATCAGCACCCAAAGCT-3′ (Frantz *et al.*, 2013) and the reverse primer *PLO-CRL1*; 5′-CGCTTAAACTTATGTCCTG-TAACC-3′ (Cullingham *et al.*, 2008). PCRs were performed using 1−10 ng DNA, 2 μL 5PRIME HotMasterMix and 0.3 μM of each primer and the amplification set-up was as follows: 3 min at 95 °C, followed by 28 cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. PCR products were purified using the High Pure PCR Product Purification Kit (Rocher, Basel, Switzerland), sequenced by Macrogen (Amsterdam, the Netherlands) and aligned in MEGA 6.06 (Tamura *et al.*, 2013).

Microsatellite genotyping

All individuals were genotyped at 16 microsatellite loci: *PLOT-01*, *PLOT-02*, *PLOT-03*, *PLOT-04*, *PLOT-06*, *PLOT-07*, *PLOT-08, PLOT-10*, *PLOT-11*, *PLOT-13* (Fike *et al.*, 2007), *PLO-M15*, *PLO-M3*, *PLO2-14*, *PLO3-86* (Cullingham *et al.*, 2006), *PLM01* and *PLM03* (Siripunkaw *et al.*, 2008). Loci *PLOT-05*, *PLO2-117, PLO-M20* and *PLO-M2* were excluded from further analysis, because they contained single steps and did not amplify in all precipitated DNA samples. We tested for significance of excess or deficiency of heterozygotes with the Markov chain method in GENEPOP 4.1.4 (Rousset, 2008), with 10,000 dememorization steps, 500 batches and 10,000 subsequent iterations. We did not observe any systematic deviations from Hardy–Weinberg expectations (HWE) at any of the remaining 16 loci. Pairs of loci were tested for linkage disequilibrium using an exact test based on a Markov chain method as implemented in GENEPOP 4.1.4. No evidence for linkage disequilibrium between pairs of loci was found.

Reactions were performed in a final volume of 10 μL containing 1−10 ng DNA, 5 μL Type-it Microsatellite PCR master mix (QIAGEN) and 0.3 μM of each primer (for PCR conditions, see Appendix S1 in Supporting Information). Hair and swab samples were amplified and scored twice to minimize the risk of genotyping errors. The fluorescently labelled PCR products were analysed on a MEGABACE 1000 automated sequencer (GE Healthcare, Freiburg, Germany), and fragment lengths of the alleles were scored by eye using FRAGMENT PROFILER 1.2 (Amersham Bioscience, Freiburg, Germany).

Genetic structure and diversity

We analysed the population genetic structure using two Bayesian genetic clustering algorithms, STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) and BAPS v.6.0 (Cheng *et al.*, 2013). In STRUCTURE, we estimated the number of genetic clusters (K) by performing ten independent runs of $K = 1$–12 for each location (Spain, Central Europe, North America) with 10^6 Markov chain Monte Carlo (MCMC) iterations after a burn-in period of 10^5 iterations, using the model with correlated allele frequencies and assuming admixture, allowing ALPHA to vary between runs. We calculated each individual's percentage of membership (q), averaging q over different runs of the most probable number of clusters based on the log-likelihood values and their convergence associated with each K. In BAPS, we performed ten runs for $K = 2$–20 for the complete data set and mapped the significant ($P > 0.05$) results of the admixture clustering of the best K using ARCGIS 10.1 (ESRI Inc., Redlands, CA, USA).

For the subsequent analyses, populations were pre-defined as given by the BAPS clustering analyses. We visualized the genetic differentiation among the samples with a factorial correspondence analysis (FCA) in GENETIX 4.05.2 (Belkhir *et al.*, 2004). We further performed genetic exclusion tests in GENECLASS 2.0 (Piry *et al.*, 2004) to test whether individuals in Central Europe and Spain can be excluded from the native population in North America. We also used this program to test for the most likely origin of individuals from zoos and the clusters that showed no clear spatial coherence (see cluster RP in results). Exclusion probabilities were calculated with the Monte Carlo method of Paetkau *et al.* (2004) by simulating 10,000 multilocus genotypes and by setting the threshold for exclusion of individuals to 0.001 (Manel *et al.*, 2002). In GENALEX version 6.501 (Peakall & Smouse, 2012), we quantified the level of genetic differentiation between clusters with F_{ST} (Weir & Cockerham, 1984) and by an analysis of molecular variance (AMOVA) using 9999 permutations. We also used GENALEX to estimate the number of alleles (A), observed heterozygosity (H_O), unbiased expected heterozygosity ($_uH_E$) for each cluster and to calculate the number of private alleles ($_pA$) in a

cluster. Allelic richness (A_R) was calculated in FSTAT 2.9.3.2 (Goudet, 2001). Incipient populations of invasive species have a high risk of inbreeding, particularly if they stem from a small number of founders. To assess the risk of inbreeding in the invasive populations of the raccoon, relatedness coefficients were calculated in COANCESTRY 1.0.1.5 (Wang, 2011), which provides the Triadic Maximum Likelihood estimator TrioML based on Wang (2007), estimating pairwise relatedness (r) by the use of a third individual as a reference, thus reducing the chance of genes identical in state being mistakenly inferred as identical by descent.

Landscape genetic analysis

The landscape genetic analysis was done only for the Central European population as this data set was larger, had a wider geographical range and a longer invasion history than the Spanish data set. We used the R-package ResistanceGA, (Peterman, 2014) to optimize a resistance surface depicting waterways. ResistanceGA is an approach to parameterize resistance surfaces to optimally fit genetic data (Richardson *et al.*, 2016), circumventing typical issues of subjectivity in assigning resistance values and the usually associated assessment of only a limited parameter space in the process of optimization and also accounting for spatial autocorrelation (Peterman *et al.*, 2014; Richardson *et al.*, 2016). Once pairwise genetic distances and coordinates of sample sites of individuals have been specified, it calls CIRCUITSCAPE to calculate pairwise resistance distances between individuals and employs a genetic algorithm to maximize fit of resistance surfaces to the specified data set, based on AIC_c values of linear mixed effects models (Shah & McRae, 2008). For the analysis, we randomly selected 247 samples to meet the pre-condition of the program, which only allows one genetic sample per surface pixel. Maps of district boundaries and waterways were created in the Geographical Information Systems ARCGIS 10.1 (ESRI Inc), using ESRI Data & Maps 2005 and the GADM database (www.gadm.org), version 2.8, November 2015. The waterway data were transformed in a grid of 500 × 500 m, using ETRS_1989_LAEA_L52_M10 projection. The three categories given by ESRI Data & Maps were considered: 'Europe Water', 'Europe smaller Major Water' and 'Europe very large Major Water'. 'Europe Water' contains a base map layer of rivers/lakes for Europe not contained in 'Europe Major Water'. 'Europe Major Water' represents the major inland water for Europe and was split in the two given types called 'European smaller major waters', which represent water bodies and province separators and 'European very large major waters', which are very large water bodies and/or country separators. Pairwise genetic distances were calculated in COANCESTRY 1.0.1.5 (Wang, 2011), using the Triadic Maximum Likelihood estimator TrioML based on Wang (2007), which was also used for the calculation of relatedness coefficients of the invasive populations. As recommended by Peterman *et al.* (2014), ResistanceGA was run twice and runs were checked for convergence by their AIC_c values. There were only marginal differences in AIC_c values between runs and no change in the ranks of the best performing factor (resistance surface of waterways, geographical distance or a null model).

RESULTS

Mitochondrial haplotype diversity of the raccoon population

Multiple introductions of raccoons were identified in both European regions (Spain and Central Europe) based upon the mtDNA data (Fig. 1). In addition to the six haplotypes (PLO2a, PLO2b, PLO16, PLO110, PLO13 and PLO75) identified by Frantz *et al.* (2013) for Central Europe, we found another haplotype (PLO57) at the Central European range margin in Rhineland-Palatinate (Germany) that was formerly reported from Minnesota (GenBank accession no: EF030370; Cullingham, 2007). We did not differentiate haplotypes PLO2a and PLO2b (which differ at nucleotide position 577) as some sequences (particularly hair and saliva samples) did not have good quality and we therefore had to cut a part at the end of the sequences. We therefore refer to haplotype PLO2 for both haplotypes here. The haplotypes PLO2, PLO13, PLO110 and PLO16 mostly represent the four main populations in Central Europe (in the German federal states HE, BB, HA and SN). Haplotype PLO75 was only found in a single raccoon captured in a suburb of the city of Luxembourg (Luxembourg) and haplotype PLO57 was observed in a single raccoon family in Rhineland-Palatinate (Germany). Haplotypes PLO2 and PLO13 (Cullingham *et al.*, 2008) are among the most common haplotypes in North America and were carried by 51.1% and 30.1% of all 229 analysed individuals in Central Europe respectively, while haplotype PLO110 was only found in raccoons in Germany so far (Frantz *et al.*, 2013). In Spain, haplotype PLO2 was found in the population in Madrid (Mad) along the Jarama River (17%), while haplotype PLO13 which was observed in the population in Andalusia (And) and south of Madrid was carried by 22.6% of all the individuals from Spain. The most common haplotype in Spain was PLO66 (35.8%), occurring in individuals from the Balearic Islands (Bal) and from Castilla-La Mancha (Cas) in Central Spain. In the native range, haplotype PLO66 was found in a raccoon from Kansas (GenBank Accession no.: EF030409; Cullingham, 2007). Haplotype PLO32 was found in Galicia (Gal), Catalonia (Cat) and an individual from Henares River in Castilla-La Mancha (Cas) in Spain (Fig. 1). Haplotype PLO32 was also found in Kansas and Minnesota (GenBank accession no: EF030359; Cullingham, 2007). However, in comparison with the results from the native range, where 76 haplotypes were found among 311 samples (Cullingham *et al.*, 2008), the mitochondrial haplotypes diversity in the invasive range is still low.

Genetic population structure of the raccoon population

The microsatellite analysis revealed 17 genetic clusters in total (in STRUCTURE; Fig. 2), with eight clusters in Central

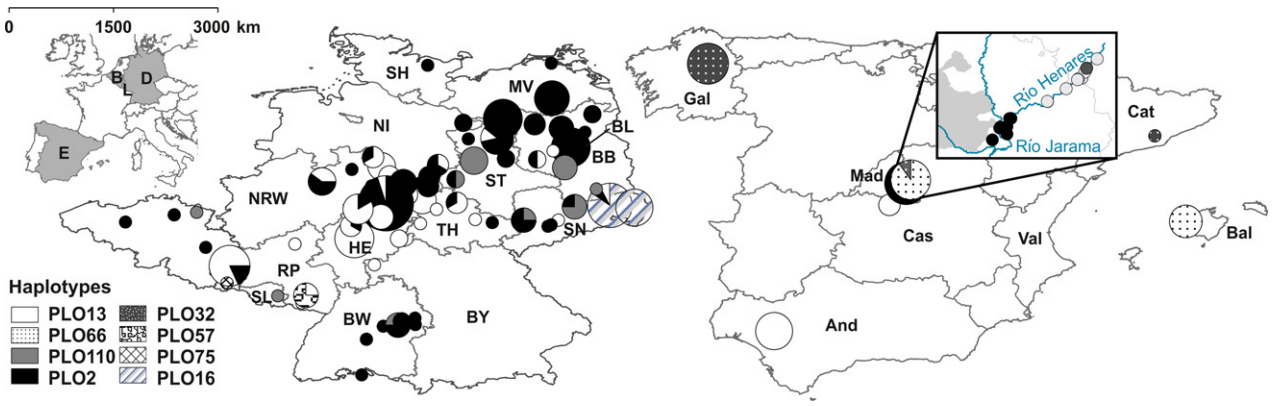

Figure 1 Geographical distribution and frequency of haplotypes observed in raccoons sampled in Europe. The inset shows the location of the four European countries sampled in this study: Germany (D), Belgium (B), Luxembourg (L) and Spain (E). The figure combines the results of Frantz *et al.* (2013) of 193 raccoons in Germany with the results of the newly analysed mtDNA samples at the range margin in Germany and those sampled in Spain. The size of the pie charts is proportional to the number of individuals sampled per locality (from 1 to 20). Grey lines are the borders of countries, German federal states (Brandenburg (BB), Berlin (BL), Baden-Württemberg (BW), Hesse (HE), Mecklenburg-West Pomerania (MV), Lower Saxony (NI), North Rhine-Westphalia (NRW), Rhineland-Palatinate (RP), Saxony (SN), Saxony-Anhalt (ST), Thuringia (TH), Schleswig-Holstein (SH), Saarland (SL), Bavaria (BY)), and Spanish provinces (Andalusia (And), Balearic Islands (Bal), Castilla-La Mancha (Cas), Catalonia (Cat), Galicia (Gal), Madrid (Mad) and Valencia (Val)) Inset: light blue lines represent main rivers, and the urban areas of Madrid are indicated in grey. [Colour figure can be viewed at wileyonlinelibrary.com]

Europe (including captive individuals), seven in Spain and two in North America (based upon the highest log-likelihood values that converged well between runs; Appendix S2). These STRUCTURE clusters are mostly consistent with the results of BAPS, which found 16 populations in the complete data set, but placed individuals from the native population in North America in a single genetic cluster (except for five individuals that were assigned to three different clusters). Two other differences in BAPS were that individuals from Luxembourg, Belgium and Rhineland-Palatinate in Germany (STRUCTURE cluster LU) were placed in the population in Hesse (HE) in Germany and individuals from STRUCTURE cluster RP were assigned to clusters covering individuals from Andalusia (And) in Spain or North America. Six of the eight STRUCTURE clusters from Central Europe were already known (HE, HA, BB, SN, LU, RP; see also Fischer *et al.*, 2015). Individuals from zoos were largely assigned to these clusters, except for two new genetic clusters that covered individuals from zoos in Saxony (cluster SNzoo) and Bremerhaven (cluster Brezoo; Fig. 2). In Spain, seven genetic clusters were found in STRUCTURE (1) in Madrid along Jarama River (Mad), (2) along Henares River in province Castilla-La Mancha (Cas), (3) in Andalusia (And), (4) in Galicia (Gal), (5) in Catalonia (Cat), (6) in Balearic Islands (Bal) and (7) in Valencia (Val) (Fig. 2). In BAPS, two of three individuals from Valencia (Val) in Spain clustered with individuals from Saxony in eastern Germany (SN) and one with cluster RP in Germany, while the two individuals from Catalonia (Cat) were assigned to the North American samples (USA, see also below). In STRUCTURE, all clusters from Central Europe had a relatively high degree of admixture, whereas admixture was nearly absent among the geographically separated populations in Spain.

Genetic similarity with the native and zoo populations

In the FCA, individuals from Central Europe were clumped and overlapped substantially with samples from the Balearic Islands and Andalusia in Spain, whereas most Spanish clusters were clearly separated (except the adjacent populations in Madrid and in Castilla-La Mancha) and had high (positive or negative) loadings on both FCA axes (Fig. 3a). The North American samples had low loadings on both axes and were thus enclosed by the Spanish ones. The populations in Spain or Central Europe did not overlap substantially with samples from the native range (except for some individuals from zoos or STRUCTURE cluster RP). In a separate FCA, including only the Central European samples (and zoo samples), a differentiation of the four main populations (HE, BB, HA, SN) was indicated along the first axis (Fig. 3b). Individuals from BAPS clusters RP, And and USA as well as from zoos and wildlife parks were distributed all over the FCA graph and formed outliers in some cases with high negative loadings on axis 2 (Fig. 3b). All seven BAPS clusters in Central Europe and five BAPS clusters in Spain (excluding clusters < 5 individuals) were strongly differentiated, with F_{ST} values ranging between 0.049 and 0.395 (Table 1a). The highest mean genetic differentiation in Central Europe was found for the captive clusters SNzoo and Brezoo (F_{ST} = 0.123–0.297). The Spanish clusters (except the adjacent clusters Mad and Cas) had a high genetic differentiation with F_{ST} ranging between 0.254 and 0.395 (Table. 1a). All non-native raccoon clusters (in BAPS), the historical and captive ones in Central Europe (HE, HA, BB, SN, RP, SNzoo, Brezoo) as well as the more recently introduced ones in Spain (Mad, Cas, Gal, And, Bal) were highly differentiated from the population in the native range

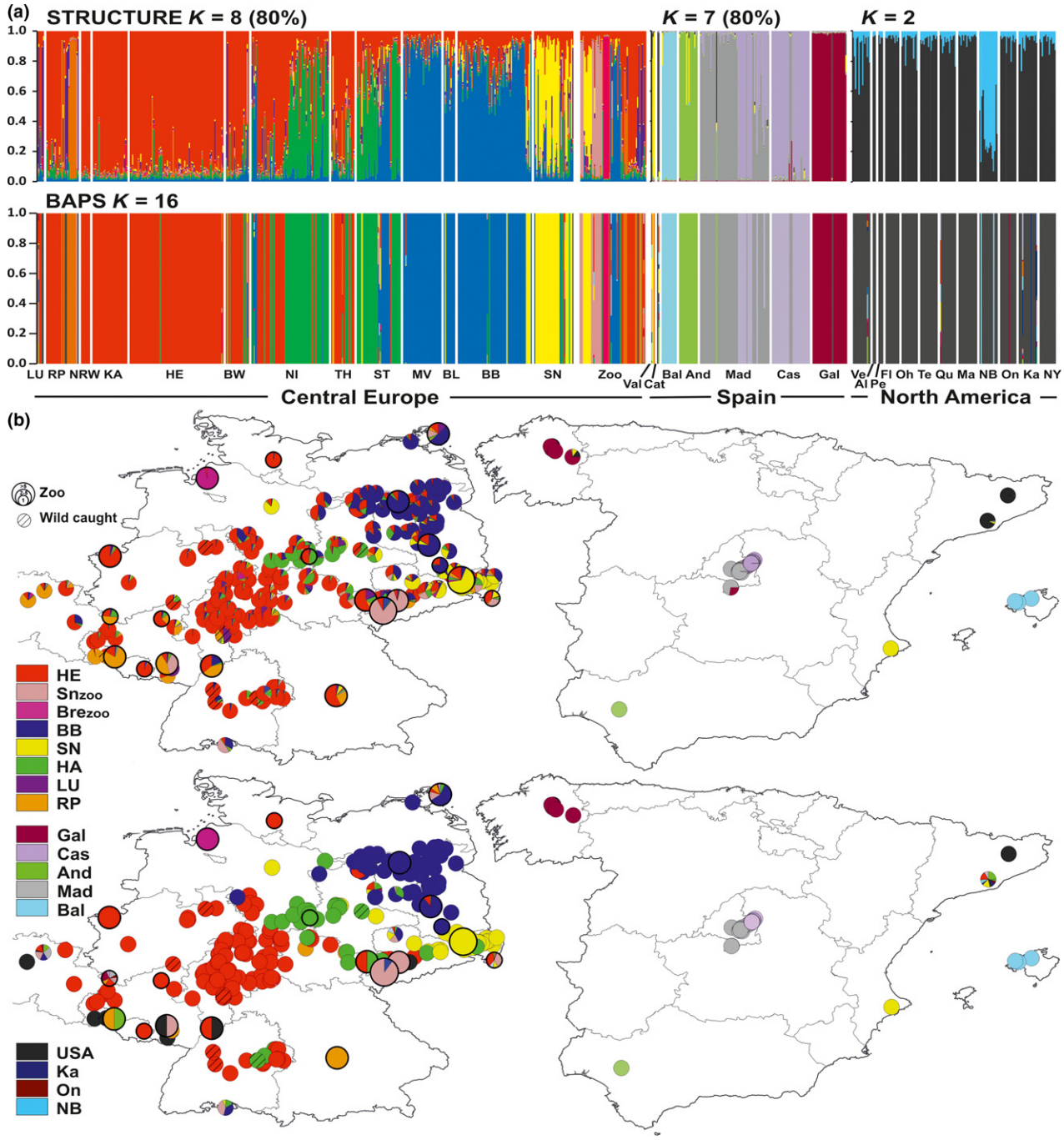

Figure 2 Geographical distribution of the genetic clusters detected in STRUCTURE and BAPS for all 806 raccoon samples. (a) The pre-defined populations correspond to the native range in North America, Central Europe, with individuals from all German zoos combined in one group and Spain. Samples are from following federal states in Germany: Brandenburg (BB), Berlin (BL), Baden-Württemberg (BW), Hesse (HE), Mecklenburg-West Pomerania (MV), Lower Saxony (NI), North Rhine-Westphalia (NRW), Rhineland-Palatinate (RP), Saxony (SN), Saxony-Anhalt (ST) and Thuringia (TH); single samples from Bavaria (BY), Schleswig-Holstein (SH) and Saarland (SL) were included in the adjacent states, from following provinces in Spain: Andalusia (And), Balearic Islands (Bal), Castilla-La Mancha (Cas), Catalonia (Cat), Galicia (Gal), Madrid (Mad) and Valencia (Val) and from following states and provinces in North America: Alabama (Al), Florida (Fl), Kansas (Ka), Maine (Ma), New Brunswick (NB), New York (NY), Ohio (Oh), Ontario (On), Pennsylvania (Pe), Quebec (Qu), Tennessee (Te) and Vermont (Ve). For Central Europe, the figure includes the previous results of Fischer *et al.* (2015) in the database of this study, which further analysed DNA samples in the margin range and the populations in German Zoos. Each individual is represented by a vertical line representing the individual's estimated proportion of membership to the genetic cluster. (b) Pie charts represent the assignment values of the admixed clustering analysis performed in STRUCTURE (above) and BAPS (bottom) for all individuals. Individuals from zoos are marked with a black margin, the size of the pie chart corresponding to the number of samples included. Individuals caught in the wild and subsequently included in the zoo population are hatched. [Colour figure can be viewed at wileyonlinelibrary.com]

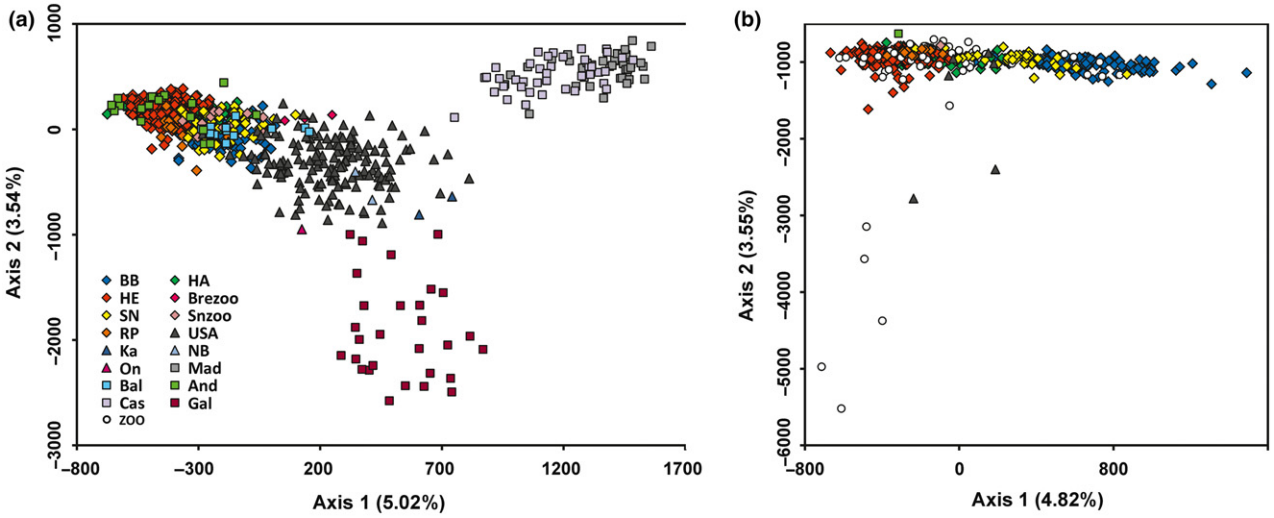

Figure 3 Factorial correspondence analysis performed for raccoons based on 16 microsatellite loci. Symbols and colours represent the different genetic clusters according to the BAPS analysis. (a) Factorial correspondence analysis with 806 samples from North America, Spain and Central Europe. (b) Factorial correspondence analysis for the 495 Central European samples (excluding outlier Lux1) and with all zoo individuals represented as a separate group (symbol: circle). [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1 Pairwise F_{ST} values (a) between all genetic clusters as identified by BAPS (b) same, but with all individuals from zoos grouped separately (c) according to the area of origin (for wild-caught individuals; CE: Central Europe, E: Spain, NA: North America) or zoo. All F_{ST} values were significant ($P < 0.001$).

(a)	HE	BB	HA	SN	RP	SN_{zoo}	Bre_{zoo}	Mad	Cas	Gal	And	Bal
BB	0.120											
HA	0.049	0.112										
SN	0.070	0.071	0.078									
RP	0.061	0.152	0.068	0.104								
SN_{zoo}	0.152	0.210	0.150	0.127	0.123							
Bre_{zoo}	0.187	0.274	0.179	0.181	0.230	0.297						
Mad	0.273	0.254	0.251	0.223	0.285	0.267	0.315					
Cas	0.252	0.243	0.232	0.209	0.269	0.268	0.265	0.101				
Gal	0.258	0.254	0.235	0.232	0.242	0.281	0.309	0.263	0.281			
And	0.112	0.183	0.120	0.161	0.129	0.200	0.298	0.305	0.313	0.304		
Bal	0.267	0.308	0.262	0.277	0.293	0.341	0.471	0.364	0.395	0.339	0.254	
NA	0.106	0.109	0.100	0.072	0.098	0.107	0.155	0.150	0.142	0.147	0.145	0.226

(b)	HE	BB	HA	SN	RP	Zoo	Mad	Cas	Gal	And	Bal
BB	0.119										
HA	0.050	0.112									
SN	0.076	0.067	0.085								
RP	0.085	0.172	0.093	0.121							
Zoo	0.023	0.082	0.038	0.032	0.060						
Mad	0.273	0.252	0.252	0.218	0.303	0.216					
Cas	0.251	0.241	0.234	0.208	0.291	0.197	0.101				
Gal	0.258	0.254	0.235	0.235	0.270	0.195	0.263	0.281			
And	0.119	0.188	0.128	0.174	0.184	0.105	0.315	0.325	0.313		
Bal	0.267	0.308	0.260	0.284	0.349	0.231	0.364	0.395	0.339	0.260	
NA	0.106	0.107	0.102	0.071	0.060	0.057	0.216	0.142	0.147	0.105	0.227

(c)	CE	E	NA
E	0.113		
NA	0.083	0.071	
Zoo	0.011	0.093	0.061

in North America (NA; F_{ST} = 0.072–0.226). If samples were grouped together according to their region of the origin, the highest differentiation was found between Central Europe (CE) and Spain (E), while both populations showed a low genetic differentiation to the population in North America (NA) (Table 1c). The AMOVA revealed that a significant part of the genetic variation (15%, $P < 0.001$) was between genetic clusters.

Due to the weak genetic structure of the population in North America, the exact source region of the European founder individuals could not be identified. Moreover, in a leave-one-out approach in GENECLASS, five individuals from Galicia (Gal19, 20, 21, 26, 28) in Spain and one individual from HA (Harz1) in Central Europe were excluded with high probability ($P < 0.001$) from the source population in North America.

Genetic diversity in the introduced, native and zoo population

Genetic diversity was low for all Spanish samples (Table 2). The average number of alleles per locus ranged from 2.4 to 3.9 (allelic richness A_R: 2.2–2.9), whereas in the native population it was 12.2 (A_R: 5.0). In Central Europe, the number of alleles per locus ranged from 4.0 to 6.4 (A_R: 3.2–3.6), while in the captive populations it was 2.1 and 3.0 (A_R: 2.1–2.8). Similar patterns were observed for heterozygosity values (Table 2). Both introduced populations in Central Europe and Spain each had one private allele ($_pA$). Considering the number of private alleles within the respective countries ($_pAb$), clusters HE and BB in Central Europe as well as Gal and And in Spain had a relative high number of private alleles. The number of private alleles in the other BAPS clusters in the non-native range varied between one and five, with no private alleles occurring in the zoo clusters. The relatedness estimates TrioML calculated in COANCESTRY indicated a high overall relatedness in the Spanish clusters, the Central European cluster RP (where a complete family of raccoons was sampled), as well as in the zoo clusters Brezoo and SNzoo (Table 2).

Rivers as pathways of gene flow

Landscape genetic results from ResistanceGA identified distance as the main predictor of gene flow based on AIC (distance: −70,411.91; waterways: −70,239.91; null-model: −65,769.22; Table 3). Despite the presence of large rivers, for example the Rhine or the Danube, no spatially congruent genetic clusters corresponding to either side of these linear features were found.

DISCUSSION

Origin and multiple introductions of the raccoon in Europe

Captive stocks of raccoons and most introduced populations usually start with a small number of founders, resulting in a genetic bottleneck during establishment. The limited number of haplotypes in Central Europe (six) and Spain (four) indicates that the raccoon in Europe is likely to originate from a small number of founders, given that mtDNA diversity is high in the natural range of the species (76 haplotypes; Cullingham *et al.*, 2008). However, as the native population structure of the raccoon in North America is rather weak, it was not possible to identify the exact source region of most European invasive populations. The haplotypes dominating in Europe (particularly haplotypes PLO2 and PLO13) are widespread in North America, except for haplotypes PLO66 and PLO57. The former has been recorded from Kansas, suggesting that the populations on the Balearic Islands and in Castilla-La Mancha in Central Spain may stem from here. Haplotype PLO57 was found in Minnesota. In the invasive range, this haplotype was only found in a raccoon family at the range margin in Rhineland-Palatinate (Germany), supporting the hypothesis that these individuals are escaped pets rather than recent dispersers from the expanding population in Hesse (Germany), which is the adjacent cluster in Central Europe. Thus, the identification of this haplotype supports the hypothesis of Fischer *et al.* (2015) that the STRUCTURE cluster RP essentially consists of recently introduced individuals, which is also indicated by the presence of individuals belonging to this cluster in several zoos as well as in geographically separated areas of Central Europe. The results from BAPS, GENECLASS, the FCA as well as the F_{ST} values support this hypothesis as well as that these individuals rather match the native population than the populations in the invasive range.

Loss of genetic diversity is often considered detrimental as it reduces the adaptability of a population and may lead to rapid population declines when the environmental conditions change (Frankham, 1995). In the two invasive ranges studied here, the raccoon shows a considerably lower genetic diversity than in the native range. However, there is no indication of any negative effects. The raccoon is a successful invader and even though genetic diversity is substantially lower than in the natural range, it still seems to be large enough to avoid any negative effects on the population. On the basis of the microsatellite analysis, we found 11 genetic clusters (in BAPS) in the non-native range (plus two in zoos), supporting the hypothesis of multiple introductions and ongoing releases of raccoons in Europe (Fischer *et al.*, 2015). Multiple introductions are known to enhance the establishment success and expansion of non-native species, as they help to overcome genetic founder effects by increasing genetic diversity, especially if the founder individuals are from several sources (Dlugosch & Parker, 2008; Schulte *et al.*, 2012). So far, the results for Central Europe of this study are in line with the findings in a previous study (Fischer *et al.*, 2015), even though we used a reduced set of microsatellite loci (16 instead of 20 loci). One exception is the cluster of individuals from the city of Kassel in Hesse (see cluster KA in Fischer *et al.*, 2015), which did not form a separate cluster in our analysis, indicating that this cluster might have been an artefact of the clustering method due to

Table 2 Comparison of genetic variability among BAPS-defined genetic clusters (excluding clusters < 5 individuals) in the different composition of the populations. (a) All identified BAPS clusters are represented (b) same, but all individuals from zoos were grouped in one separate cluster. (c) All individuals were sorted according their respective area (CE: Central Europe, E: Spain, NA: North America) or the zoo cluster.

Site	N	A	A_R	H_O	${}_uH_E$	${}_pA$	freq$_pA$	${}_pAb$	freq$_pAb$	*TrioML*
(a)										
HE	201	6.4	3.2	0.57	0.59	1	0.005	9	0.002–0.005	0.244
BB	125	5.8	3.4	0.51	0.56	0		6	0.008–0.108	0.243
HA	82	5.8	3.5	0.60	0.63	0		2	0.006–0.012	0.208
SN	45	5.6	3.6	0.60	0.62	0		1	0.011	0.173
RP	18	4.0	3.2	0.56	0.59	0		2	0.111–0.278	0.293
SN_{zoo}	12	3.0	2.8	0.69	0.61	0		0		0.452
Bre_{zoo}	5	2.1	2.1	0.60	0.44	0		5	0.200–0.600	0.668
Mad	50	3.9	2.9	0.58	0.58	0		5	0.010–0.190	0.434
Cas	42	3.5	2.9	0.59	0.55	0		4	0.012–0.098	0.441
Gal	29	3.2	2.8	0.63	0.59	1	0.536	14	0.018–0.536	0.455
And	19	3.8	2.8	0.59	0.55	0		12	0.028–0.523	0.418
Bal	13	2.4	2.2	0.40	0.40	0		3	0.115–0.885	0.622
NA	160	12.2	5.0	0.73	0.77	51	0.003–0.078			0.087
(b)										
HE	188	6.3	3.8	0.57	0.59	1	0.005	10	0.003–0.024	0.240
BB	117	5.8	4.3	0.51	0.56			6	0.009–0.098	0.240
HA	79	5.7	4.4	0.60	0.62			2	0.006–0.135	0.201
SN	37	5.4	4.5	0.60	0.61			1	0.014	0.179
RP	12	3.5	3.4	0.55	0.52			2	0.042–0.292	0.382
Zoo	57	6.8	5.1	0.58	0.66					0.122
Mad	50	3.9	3.2	0.58	0.57			5	0.010–0.190	0.434
Cas	42	3.5	3.2	0.59	0.54			4	0.012–0.098	0.441
Gal	29	3.2	3.1	0.63	0.58	1	0.536	14	0.018–0.536	0.454
And	18	3.7	3.3	0.59	0.52			12	0.028–0.523	0.443
Bal	13	2.4	2.4	0.40	0.38			3	0.115–0.885	0.621
NA	158	12.2	7.0	0.73	0.76	51	0.003–0.078			0.086
(c)										
CE	439	8.3	6.5	0.57	0.64	1	0.002			0.146
E	157	7.6	7.0	0.58	0.71	1	0.096			0.185
NA	153	12.3	10.5	0.73	0.77	47	0.003–0.072			0.090
Zoo	57	6.8	6.8	0.58	0.66					0.125

N = sample size. A = average number of alleles per locus. A_R = allelic richness (based on a minimum sample size of five diploid individuals). H_O & ${}_uH_E$ = observed and unbiased expected heterozygosities. ${}_pA$ = number of private alleles in the data set. freq$_pA$ = frequency range of private alleles. ${}_pAb$ = number of private alleles within the respective area. freq$_pA$ = frequency range of private alleles in the respective area. *TrioML* = average relatedness estimate.

Table 3 Results of landscape genetic analysis using ResistanceGA. The waterways model explains less of the genetic variation encountered in Central European raccoons than sole geographical distance between individuals.

Surface	AIC_c
Distance	−70,411.91
Waterways	−70,239.91
Null-model	−65,769.22

closely related family lineages included before. Our data from Spain support the hypothesis of recent introductions of individuals leading to geographically separated and genetically distinct populations.

Genetic similarity of the Central European, Spanish and North American populations

We detected a major difference in the degree of genetic differentiation between the populations in Spain and Central Europe (Fig. 3). The Central European populations are genetically more similar than the Spanish populations, suggesting that either the founders were genetically less differentiated or that recent admixture decreased the level of differentiation. Given that the Central European populations have established during the last 80 years (which means 40–80 generations) and that the species is quite dispersive, a genetic homogenization caused by genetic admixture is not surprising. By contrast, the plot of the genetic distances based on the microsatellites (Fig. 3) corroborates the

distinctive genetic composition of the Spanish populations. As individuals in Spain had a high degree of relatedness within the clusters, it will become interesting to study whether inbreeding may negatively affect the young Spanish populations in the future. However, the incipient contact between the two subpopulations from Madrid and Castilla-La Mancha at the River Henares in Spain (Fig. 2, Alda *et al.*, 2013) and periodic introductions of new individuals in this area may compensate for this process (Dlugosch & Parker, 2008; Witzenberger & Hochkirch, 2011). Strong genetic structure is typical of introduced populations at early stages of invasions (Schulte *et al.*, 2013), but the high genetic diversity of recently founded raccoon populations in Spain might raise the genetic diversity in the future.

Captive populations of invasive species

Accidental or deliberate releases of household pets or individuals from zoos are fairly common and known to have caused several invasions (Hulme *et al.*, 2008). The raccoon is one of these species and it has been suggested that some small, isolated feral populations were integrated into the larger invasive populations during range expansion, increasing the invader's genetic potential (Alda *et al.*, 2013; Fischer *et al.*, 2015). Almost all samples from captive stocks in Germany were part of the same genetic cluster as the wild-caught individuals in the surroundings, indicating an extensive exchange. These common genetic clusters are probably mainly caused by integration of wild-caught individuals into zoo populations. However, some are also caused by recent escapees or releases. On the other hand, some zoo populations are quite isolated, for example the highly related individuals of the zoo in Bremerhaven (Brezoo) as well as the zoos in Saxony (SNzoo) formed distinct genetic clusters. While the SNzoo individuals had no private alleles and overlapped with clusters SN and BB (from Germany) in the FCA, Brezoo individuals had a high number of private alleles and a close relationship to the North American clusters in the FCA. Therefore, there is even a potential to increase the genetic diversity of the invasive populations in case of a future escape.

Given that periodic introductions of new individuals are a problem in both European regions, control of pet keeping and trade is essential in management efforts concerning this species. As the 'polluter-pays' principle is generally considered an efficient instrument in environmental policy, especially for its deterrent and preventive effect, genotyping commercial stocks might be a possibility for limiting illegal or accidental releases and subsequent range expansions.

The role of rivers

Previous studies found that dispersal of the raccoon is strongly affected by the spatial distribution of resources, such as food, water availability and den sites (Gehrt & Fritzell, 1998) and that mammal movements are frequently influenced by habitat connectivity or landscape resistance (Coulon *et al.*, 2004; Cushman *et al.*, 2006). Our landscape genetic analysis did not support the hypothesis that rivers influence the genetic structure. In the native range, rivers are considered biogeographical features with differential permeability for raccoon gene flow and diseases shaping raccoon population structure (Cullingham *et al.*, 2008, 2009; Rees *et al.*, 2008; Côté *et al.*, 2012). Our results for north-west and Central Spain show that rivers may represent habitat corridors as proposed by Alda *et al.* (2013), given that most samples were collected in a distance of less than 100 m from rivers. However, as sampling effort was biased and focused on rivers, such conclusions have to be drawn with caution. As spatial and temporal scales affect the spatial genetic structure (Anderson *et al.*, 2010; Spear *et al.*, 2010), we also presume that the scale of the study is important to determine the effect of waterways. While we tested waterways at a very large scale in Central Europe and found a much stronger effect of distance at this scale, this does not rule out waterways as an important corridor for dispersal at the local scale. Furthermore, it must be taken into consideration that the invasive populations are not in equilibrium yet and the overarching effect of multiple invasions may still overlay any structure caused by landscape features.

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SUPPORTING INFORMATION

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

Appendix S1 PCR conditions.

Appendix S2 Log-likelihood values.

Appendix S3 Names of people and institutions providing samples.

BIOSKETCH

Marietta L. Fischer is interested in invasive species and conservation biology and her research has focused on the processes of colonization and consequences of the raccoon in Germany.

Author contributions: M.L.F. and A.H. designed the study and analysed the data. M.L.F. generated the genetic data. M.L.F. and A.H. wrote the paper, with contributions from the other authors. J.B. conducted the landscape genetic study. R.K. supervised M.L.F. All others authors contributed significantly to sample collection.

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