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ORIGINAL PAPER

Going west—invasion genetics of the alien raccoon dog *Nyctereutes procynoides* in Europe

Christian Pitra · Sabine Schwarz · Joerns Fickel

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Abstract The raccoon dog, a medium-sized carnivore, has long been recognised as a prominent example of an invasive alien species in Europe with a wide distribution, significant ecological impact and remarkable dynamics of spread at both national and continental scales. We conducted a study of genetic diversity of 73 individuals collected at 20 sites across North and Central Europe to (1) identify major phylogenetic lineages and (2) elucidate spatial patterns of population genetic structure. Reconstructed phylogenies reveal two major clades differing on average by Tamura–Nei corrected distance of 3.4% for a 599-bp segment of the mitochondrial control region corresponding to a coalescence time of approximately 457,800 years ago (95% CI, 223,300–773,900). Many expectations based on introduction history, such as the presence of signatures of repeated founder effects and subsequently rapid population expansion, were not confirmed by our demographic analyses, probably due to an insufficient amount of time since translocations. Nevertheless, global F_{ST}=13.9% and landscape approaches provided evidence for weak population genetic structure that followed a pattern of isolation by distance. Finally, we found no congruence between previously reported morphological differentiation and the sorting of mtDNA variation. We therefore conclude that an exceptional combination of factors including multiple translocations, secondary contact and admixture of divergent matrilineages, as well as natural processes of colonisation associated with a wide ecological tolerance, promoted the successful spread of the raccoon dog into Europe.

Keywords *Nyctereutes* · Invasion · Mitochondrial DNA · Control region · Population genetics · Europe

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Introduction

Invasive alien species are often a significant threat to ecosystems worldwide and as such attract increasing attention (Clavero and Garcia-Berthou 2005). Species that have been proven to be successful invaders over a large geographical area have received most of the attention (Weber 2003). In Europe, *Nyctereutes procynoides* has long been recognised as a prominent example of an invasive alien with a wide distribution, significant ecological impact and remarkable dynamics of spread at both national and continental scales (Nowak 1973; Kauhala 1996).

The raccoon dog lineage diverged from the other canids probably as early as seven to ten million years ago (Wayne 1993). The evolutionary history of this lineage is filled with successive radiations repeatedly occupying a broad spectrum of niches, ranging from large pursuit predators to small omnivores or even frugivores. During the Pliocene, the genus was spread all over Eurasia with three species in Europe (*Nyctereutes donnezani*, *Nyctereutes tingi* and *Nyctereutes megamastoides*) and two in Asia (*N. tingi and Nyctereutes sinensis*; Dermitzakis et al. 2004). *Nyctereutes* became extinct in Europe already before the beginning of

the Pleistocene, whilst in Asia, they persisted until the present day with the living species *N. procyonoides*. This species is believed to originate from *N. sinensis* (Tedford and Qiu 1991). Nowadays, *N. procyonoides* is a native of East Siberia, East Mongolia, China, North Vietnam, Korea and Japan (Ward and Wurster-Hill 1990; Nowak 1993). Our understanding of raccoon dog systematics has long been limited by inadequacy of historical type material and an overestimation of the impact of geography on taxonomy. Consequently, the validity of the currently recognised five to six subspecies is debated in the scientific community (Sheldon 1992; Ward et al. 1987).

From 1929 to 1955, approximately 9,100 raccoon dogs were introduced as fur game species into several locations of the former Soviet Union (i.e. West Russia, the Ukraine, Siberia, Kazakhstan, Kyrgyzstan and the Caucasus region) (Lavrov 1971). Although the precise origin of the primary introduced animals is quite unknown, they most likely descended from populations of the subspecies *Nyctereutes procyonoides ussuriensis* in the Amur-Ussuri region of Far East Russia (Morozov 1953; Ralli and Kritskaya 1953). From primary introduction sites in northwest (NW) Russia and the Ukraine as well as by escaping from fur farms, raccoon dogs soon began to colonise neighbouring countries to the north and west. In the period from 1935 to 1984, raccoon dog has expanded its range at an average annual rate of 40 km and colonised 1.4 million square kilometres of Europe (Nowak 1984; Helle and Kauhala 1991). They first invaded Finland in 1935, reached Sweden in 1945–1946, then Romania in 1952, Poland in 1955, Slovakia in 1959, Germany and Hungary in 1961–1962 and Norway in 1983 (Nowak and Pielowski 1964; Kauhala 1996). *N. procyonoides* is now abundant and common throughout Finland, Poland, Belarus, Latvia, Lithuania, Estonia, the Ukraine, western Russia and Germany. It occurs also in the Czech Republic, Slovakia, Hungary, Bulgaria, Moldova and Romania. It is sporadically seen in Sweden, Austria, Bosnia, France, The Netherlands, Denmark, Norway, Slovenia and Switzerland (Mitchell-Jones et al. 1999; Kauhala and Saeki 2004). Recently, the raccoon dog has reached Macedonia and Italy (Cirovic 2006; Lapini 2006). It can be deduced from its range expansion history that the raccoon dog will continue its expansion in Europe and will increase in numbers in some areas where populations have been established (Sutor 2008). Moreover, the invading raccoon dogs may benefit from the warming of climate because long winters appear to limit their distribution, as shown in Finland (Helle and Kauhala 1991).

Despite substantial efforts to understand the ecology, history and impacts of raccoon dog invasions, there has been no comprehensive examination of the genetic patterns associated with its well-documented range extension. Understanding the broader aspects of the species' evolutionary and invasion history has been hindered by a lack of comprehensive sampling and appropriately informative genetic markers.

In the present study, we used sequence data of the mtDNA control region to evaluate (1) the extent and distribution of genetic variation within and among introduced populations and (2) the potential genetic consequences of its large range and recent history of range and population expansion. We specifically aimed to infer the phylogenetic patterns of the introduced raccoon dogs in order to determine the effect of multiple native-range sources for within-population genetic variation in introduced populations. Given the complex history of anthropogenically influenced range expansion that began in the first half of the twentieth century with the establishment of the first known non-native population in NW Russia, we searched for any traces of isolation by distance patterns of natural dispersal during the colonisation process of this species. Finally, we relate the results of the molecular analyses to previously documented morphological changes among non-native raccoon dog populations and discuss how different genetic markers can complicate the search for general patterns across invasion events.

Materials and methods

Sampling

Raccoon dogs are elusive small carnivores that remain extremely difficult to obtain samples from due to their dominantly nocturnal way of life (Nowak 1993). A total of 73 raccoon dog individuals were sampled from 20 localities across their invading range in Europe, including Finland and Germany (Fig. 1 and Table 1). Despite all efforts, our sample collection is not exhaustive due to difficulties in obtaining samples from some parts of Europe (e.g. Poland and Ukraine). We therefore had to take a conservative approach to the grouping and interpretation of our data. Raccoon dogs in Europe live in mixed agricultural landscapes (Drygala et al. 2008), but inhabit also areas of forested streams or river valleys and areas surrounding lakes where thick underbrush, marshes or reed beds provide dense cover (Ward and Wurster-Hill 1990; Nowak 1993). This has enabled us to group our samples into three of the major European drainage areas: the Lake Päijänne in Finland and the Oder and Elbe river basins in Germany. Populations and sites from which less than five individuals had been sampled were excluded to increase robustness of the analysis. Sample tissues were obtained from skin, ear, liver and spleen and preserved in 100% ethanol as soon as possible after collection. A list of accession numbers of all specimens used in this study is available from the senior author.

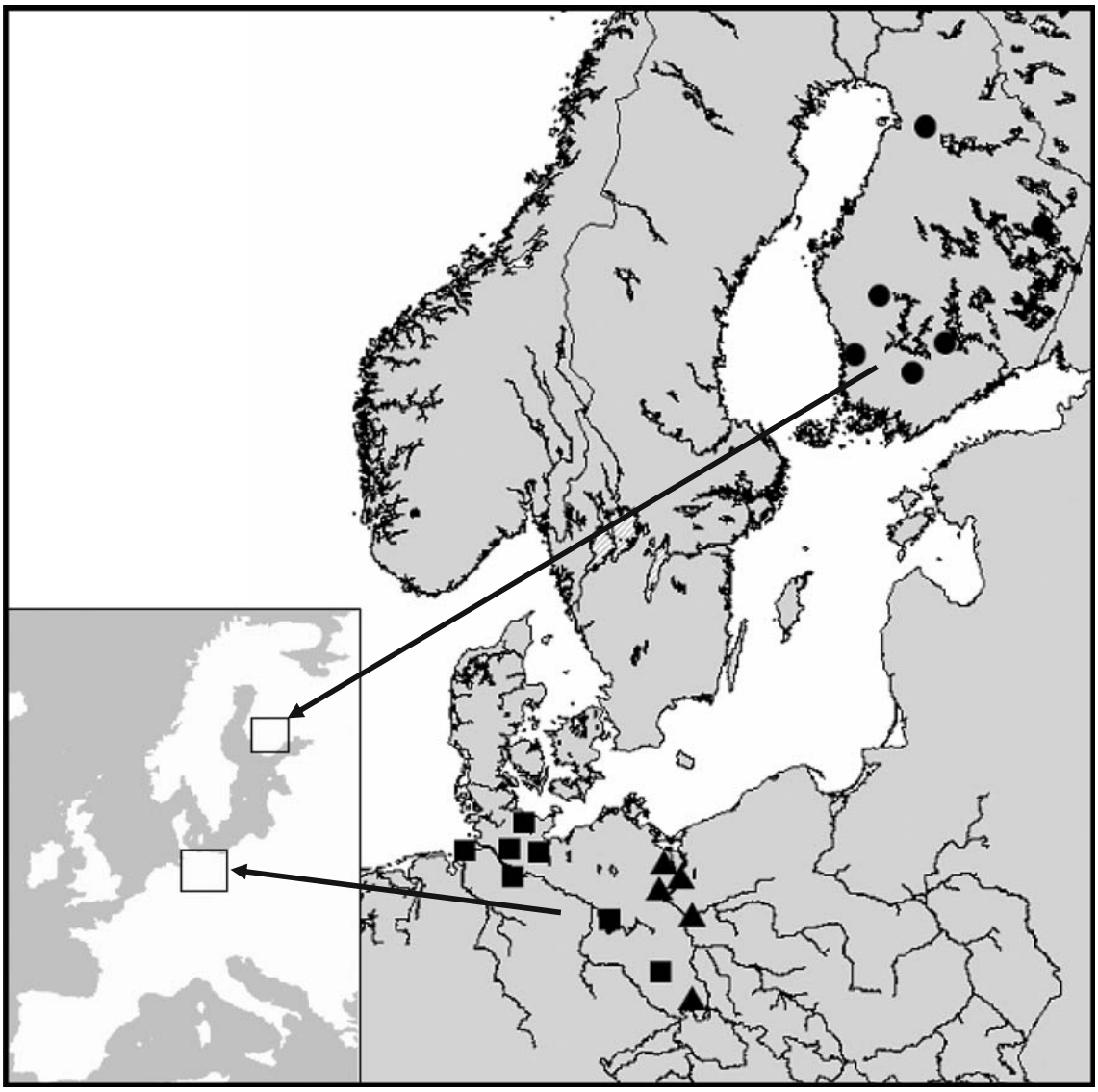

Fig. 1 Map of sampling localities for the invading raccoon dog *N. procyonoides* in Europe (*inset*). Only the sampling sites of the drainage area of the Lake Päijänne in Finland (*black circles*), the Oder river basin (*black triangles*) and the Elbe river basin (*black squares*) in Germany are shown for the sake of clarity. See Table 1 for detailed information on sampling locations

DNA isolation and sequencing

Total genomic DNA was extracted from all specimens using a DNeasy tissue extraction kit (QIAGEN) following the manufacturer's protocol. A 599-bp region of the hypervariable segment I of the control region of the mtDNA (positions 15457-16010; GenBank accession no. DQ480508; Bjornerfeldt et al. 2006) was amplified in 73 *N. procynoides* individuals. Samples were amplified using primers ProL (Palumbi et al. 1991) and MaH-CTR660R (5′-tgtgtaaaagttcttatgtcc-3′). Amplifications were performed in 1× AmpliTaq buffer (Applied Biosystems), 2 mM $MgCl_2$, 0.2 mM dNTP each, 0.4 µM each primer, 0.2 U AmpliTaq polymerase and 10–50 ng DNA. Amplification conditions were an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, with a final extension of 10 min at 72°C. Sequencing was performed using the ABI Prism™ Big Dye Terminator Cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Products were sequenced on an ABI 3130*xl* automated DNA sequencer. SeqEd (version 1.0.3, Applied Biosystems) was used to edit chromatogram files to determine bidirectional consensus sequences and to manually align sequences across samples.

Sequences were verified as *Nyctereutes* mtDNA using the GenBank *blastn* search algorithm (Altschul et al. 1990)

Table 1 Variable sites for the control region (mtDNA) surveyed in raccoon dogs (total length 599 bp) and distribution of clades I and II haplotypes by main regions. A summary of statistic parameters and tests of population growth are shown. Positions correspond to positions of the grey wolf (*Canis lupus*) mtDNA genome (GenBank DQ480508). The "–" represents a gap and "." matches the nucleotide in the first sequence. *n.p.* no analysis performed

Clade	Haplotype	Accession number	65012656482293118362 59333344555714499024 55666666667788999000 55555555555555555666 11111111111111111111	Population					
				Finland	Oder	Elbe	Weser	Danube	Rhine
	H_1	FJ888513	ATATCGGACTTCTTGTTGAG	0	20	5	0	1	1
I	H_2	FJ888514	...C....TC.....	4	5	3	1	1	0
	H_3	FJ888515	.....A	1	4	1	0	2	0
	H_4	FJ888516	.....-....A	2	5	0	1	0	0
	H_5	FJ888517	...C....TC....-.....	6	0	0	0	0	0
	H_6	FJ888518	...C....TC....-..A..	1	0	0	0	0	0
	H_7	FJ888519	...C....T.....	0	1	0	0	0	0
II	H_8	FJ888520	GCG.TAAG..CACA-AC.GA	1	1	2	0	0	0
	H_9	FJ888521	GCG.TAAG..CACA.AC.GA	3	1	0	0	0	0
			No. individuals	18	37	11	2	4	1
			Gene diversity	0.837± 0.057	0.676± 0.073	0.746± 0.098	n.p	n.p	n.p
			Nucleotide diversity	0.013± 0.007	0.005± 0.003	0.011± 0.006	n.p	n.p	n.p
			Mean number of pairwise differences	7.774± 3.800	3.000± 1.593	6.655± 3.403	n.p	n.p	n.p
			Tajima's D statistic	0.666 P > 0.10	-1.137 P > 0.10	0.117 P > 0.10	n.p	n.p	n.p
			Fu's Fs statistic	3.436	1.260	4.395	n.p	n.p	n.p
			R2 statistic	0.170	0.080	0.175	n.p	n.p	n.p

to confirm the absence of nuclear copies (e.g. pseudogenes) or other unintended sequence types. All unique sequences were deposited with GenBank (accession nos: FJ888513–FJ888521).

Analysis of mitochondrial DNA sequence data

We used DNASP (v.4.20.2; Rozas et al. 2003) to calculate the number of polymorphic sites, the average number of pairwise differences, haplotype diversity (h) and nucleotide diversity (π) as well as to compare the demographic history of populations and haplogroups using a variety of estimators based on the coalescence theory. First, signatures of old demographic population expansion were investigated for mtDNA using pairwise mismatch distributions (Harpending 1994). The goodness-of-fit of the observed data to a simulated model of expansion was tested with the raggedness (r) index (Rogers and Harpending 1992), which gives higher values for stable populations and lower values for expanding populations. Ramos-Onsins and Rozas (2002), Tajima and Tajima (1989), Fu's (1997) F_S tests of selective neutrality as well as Fu and Li's (1993) D^* and F^* test statistics were likewise performed using DNASP. The software ARLEQUIN (v.3.0; Excoffier et al. 2005) was used to calculate pairwise F_{ST} measures between sites and to compare genetic differentiation among geographical regions using analysis of molecular variance (AMOVA; Excoffier et al. 1992). Additionally, we used the ALLELES IN SPACE (AIS) program package (Miller 2005) to perform several analyses based on the georeferenced individuals (haplotypes). The landscape approach in AIS does not require prior assumptions of population boundaries. To test for isolation by distance (IBD), Mantel tests (Mantel 1967) were performed to estimate the correlation between genetic and geographical distances of observations across the landscape. To determine the probability of observing a correlation coefficient greater than or equal to that computed from original data, a set of 5,000 replicates was generated. AIS was also used to infer the presence and extent of genetic structure using spatial autocorrelation analysis (Sokal and Oden 1978). AIS performs a slightly unconventional form of a generalised spatial autocorrelation analysis calculating the simple Ay statistic. Ay can be interpreted as the average genetic distance between pairs of individuals that fall within distance classes. P values for each distance class are obtained via a randomisation

procedure consisting of 3,000 replicates to identify distance classes where average genetic distances were significantly larger or smaller than random expectations.

Phylogenetic relationships among mtDNA sequences were assessed using maximum likelihood (ML), maximum parsimony (MP), and neighbour joining (NJ) approaches implemented in PAUP* (v.4.0b10; Swofford 2001). Gaps (insertions/deletions) were coded as a fifth base in all analyses. MODELTEST (v.3.7; Posada and Crandall 1998) was used to determine the appropriate model parameters for ML analysis. ML bootstrap values were determined with 100 replicates (Felsenstein 1985) using the same settings as for the heuristic search. MP analyses used the heuristic search option with tree-bisection-reconnection branch swapping and random additions of taxa, collapsing zero-length branches and equal weighting of all characters. NJ analysis was performed using a heterogeneous model of evolution that allowed for different parameters for each data partition. MP and NJ tree topologies reliability was assessed by 1,000 bootstraps.

Estimates of divergence time and their 95% confidence intervals (CI_{95}) were then obtained by the method of Haubold and Wiehe (2001) implemented in the software program CITE (accessible at <http://gump.auburn.edu/srsantos/cite/>). This method assumes that the number of nucleotide substitutions and the substitution rate for a particular pair of taxa may be different from any other pair, and they are modelled according to a gamma distribution. The calibration point of the reference pair was based on the split between wolf and coyote at one million years ago which corresponds to a sequence divergence of 7.5±0.2% per million years as estimated by Vilà et al. (1997) on the basis of the complete mitochondrial control region.

Results

Phylogenetic analyses

Given that outgroup taxon permutations did not influence ingroup topology (data not shown), we only presented phylogenetic trees rooted with the homologous sequence of the sister taxon *Nyctereutes procyonoides viverrinus* (GenBank: D83614; Okumura et al. 1996). With this outgroup included in the analysis, there were 41 polymorphic sites. Of these, 19 were variable among the 73 *N. p. ussuriensis* sequences (Table 1). The mean distance between sequences from both subspecies was 4.5% (range 3.8–5.5%). In total, nine haplotypes were found in our dataset with a sequence divergence of 0.2–3.2% (mean 1.3%). MODELTEST identified the TN93 substitution model (Tamura and Nei 1993) + rate heterogeneity [−ln L=1395.08 (AIC); base frequencies set to A=0.2657, C=0.2845, G=0.1646, T=0.2852; Ti/tv ratio=3.8815; I=0.6679; Γ=0.1312] as the best fitting model for the data. All phylogenetic analyses (NJ, MP, ML) provided congruent tree topologies (Fig. 2). Most surprisingly, the trees split European raccoon dogs into two relatively well-supported clades (bootstrap support >83%; Fig. 2). The clades were reciprocally monophyletic and represented by either seven haplotypes (clade I: H_1–H_7) or two haplotypes (clade II: H_8–H_9; Table 1). The Tamura–Nei corrected sequence divergence±standard error between clades was 3.43±1.06%. Average sequence divergence was low in clade II (0.1±0.09%), whilst it was higher in clade I (0.38%±0.19%), which has more haplotypes that also cover greater geographic distances. AMOVA indicated that only 16.1% of the variation was due to *within*-clade variation, whilst 83.9% of the variation came from *between*-clades variation (p<0.005). Based on the calibration for the canid mtDNA control region, the two clades diverged approximately 457,800 years ago (CI_{95}, 223,300–773,900).

Historical demography

Once we had identified a pre-colonisation partition event into two different lineages, which we assume is the main determinant of genetic structure in non-native raccoon dogs, the demographic histories of clades I and II were compared using a variety of estimators based on the coalescence theory (Kingman 1982). A priori inferences of population processes were based on tests of neutrality (Tajima 1989; Fu and Li 1993; Fu 1997) that assume a constant population size, no recombination and no migration. Overall, neutrality tests in Table 2 show positive values, but they were not statistically significant. These results are not consistent with a demographic expansion from a more restricted distribution to the current range. Demographic histories were also inferred by a pairwise mismatch distribution analysis using Rogers and Harpending (1992) model of population expansion. This analysis, when applied to the whole dataset, showed a multimodal mismatch distribution, seen in populations with relatively constant size (data not shown). As the pooling of highly differentiated samples (i.e. clade I plus II) may introduce bias and because there were too few samples available for clade II to obtain reliable results, a separate mismatch distribution analysis was performed for clade I alone. This sample was characterised by a narrow left skewed unimodal mismatch distribution, a low mismatch mean of 2.1 and a very small raggedness statistic (r=0.024), suggesting a population expansion for this clade (Fig. 3 and Table 2). This assumption was supported by the ratio of θ_0 and θ_1, estimators that reflect the clade size prior to and post-expansion ($\theta_0/\theta_1 = 3.6 \times 10^3$). However, the sums of squared deviations of mismatch distribution under the population expansion model were not significant for the clade I group (SSD=0.008, p=0.66).

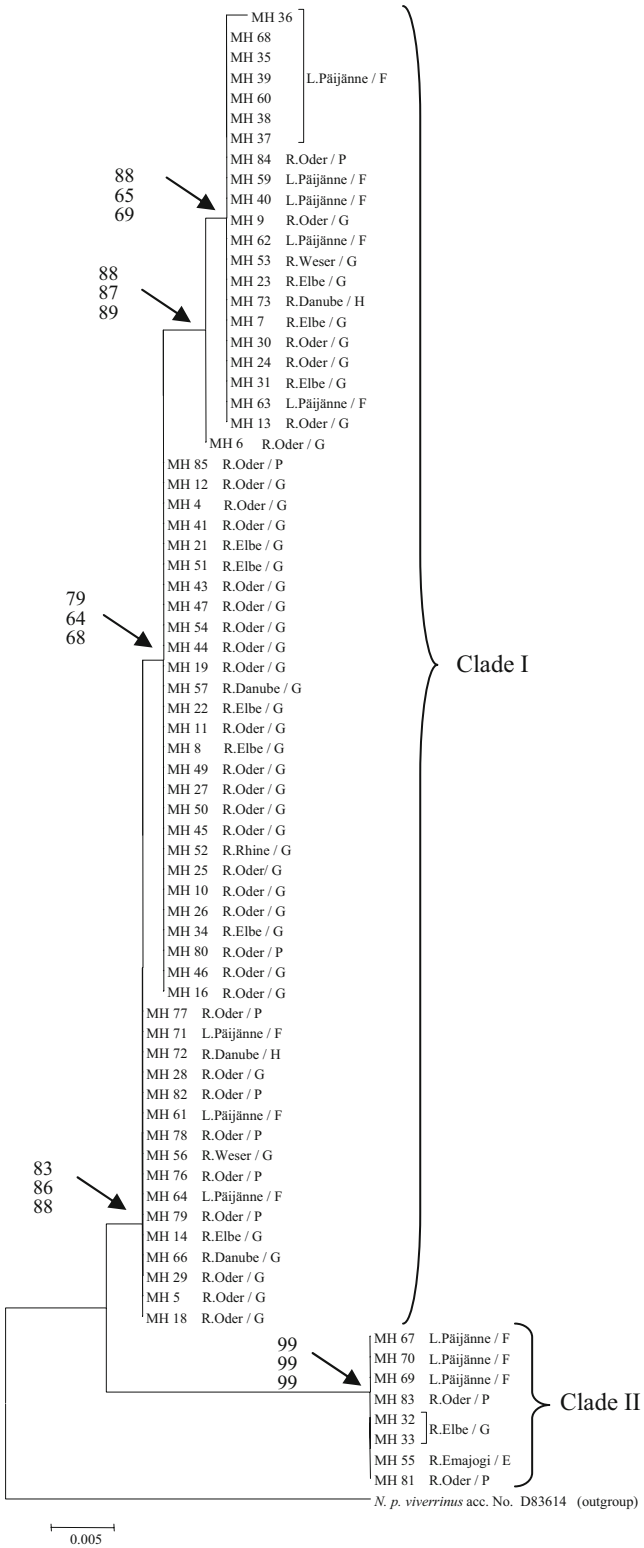

Fig. 2 Majority rule consensus tree resulting from the maximum-likelihood analysis of 599 bp of mtDNA for *N. p. ussuriensis* and an outgroup (*N. p. viverrinus*). Numbers of included specimens, population assignment and geographic origin of raccoon dog sequences are given as follows: *F* Finland, *E* Estonia, *P* Poland, *G* Germany and *H* Hungary. Clade support values for each node are bootstrap values of maximum-likelihood on *top*, maximum parsimony in the *middle* and neighbour-joining analysis at the *bottom*. *Brackets on the right* indicate the phylogenetic distinct clades I and II

Spatial analyses of phylogeographic structure

In contrast to the pronounced phylogenetic differentiation, invading populations obviously lacked geographic variation (Table 1 and Fig. 2). AMOVA analysis revealed a low but significant structure between populations of the Lake Päijänne drainage area in Finland and the geographically distant Oder and Elbe river basins in Germany (only 13.9% of the variance was assigned to the difference among those regions, $p<0.001$). Most of the total genetic variance was found within populations. The population from Finland was more similar to the river Elbe ($F_{ST}=0.14$, $p=0.004$) than to the river Oder population ($F_{ST}=0.21$, $p<0.0001$), respectively. These results are consistent with a potential invasion corridor, connecting the Finnish population with the German river basins via the coastline of the Baltic Sea (Schwarz et al. 2004). However, the presence of haplotypes H_1 and H_7 in Germany, but not in Finland, is significant because it implicates a second introduction route to Central Europe that had so far not been identified (Table 1).

In order to avoid the potential bias that could stem from either an arbitrary definition of populations and/or deviations from the mutation-drift equilibrium seen in recently invading populations (Slatkin 1993), we inferred spatial genetic patterns at an individual level of georeferenced haplotypes. The following tests used individuals/haplotypes as the operational units and were based on the exact sampling location of the individuals/haplotypes. The Mantel test based on the pairwise genetic and geographic distances of observations across all collection localities showed a low but significant ($r=0.251$, $p=0.001$) correlation between these two parameters. A relatively high regression coefficient was also obtained if we used the haplotypes found in Finland ($r=0.427$, $p=0.002$). Contrary to the Mantel test result obtained for the Finnish haplotypes, there was no significant association between genetic and geographic distances for the individuals of the combined Oder and Elbe River basin ($r=0.047$, $p=0.240$).

The spatial autocorrelation analyses using seven distance classes yielded similar results (Fig. 4).

Over the first shortest distance class (encompassing geographic distances up to 500 km), the whole European dataset yielded highly significant values of $Ay=0.17$ ($p=0.004$) that were smaller than random expectations ($Ay=0.245$) The distance classes 5 and 6 of this dataset were significantly higher than random at the $\alpha=0.05$ level ($p=0.003$). The last distance class has the highest autocorrelation coefficient, but it was not significant ($Ay=0.468$, $p=0.09$), presumably due to the smaller sample size (11). One could argue that the clade II haplotypes have induced a bias in the results because of their

Table 2 Results of historical demographic analyses of clade I and clade II groups of *Nyctereutes* in Europe

Statistic	Clade 1	Clade 2
Diversity estimates		
Number of sequences	65	8
Number of polymorphic sites	6	1
Total number of mutations (η)	6	1
Number of pairwise differences	2.122±2.884	0.571±0.254
Number of haplotypes, h	7	2
Nucleotide diversity (π)	0.00354	0.00095
Haplotype (gene) diversity	0.753±0.001	0.571±0.010
Mismatch distribution analysis		
Mismatch observed mean	2.122	0.571
Raggedness statistic (r)	0.024	0.347
Tau	1.249	0.571
Theta initial (θ_0)	0.001	0.000
Theta final (θ_1)	3.668	
Sum of squared deviation	0.008	
p value of sum of squared deviation	0.656	
Tests of selective neutrality		
Ramos-Onsins and Rozas R^2 statistic	0.176	0.286
Tajima's D statistic	1.640, p>0.10	1.444, p>0.10
Fu and Li's D* test statistic	0.256, p>0.10	0.888, p>0.10
Fu and Li's F* test statistic	0.826, p>0.10	1.100, p>0.10
Fu's F_S statistic	0.910, p>0.10	0.966, p>0.10

greater genetic distance from all other haplotypes. After removing these haplotypes from the dataset (i.e. by analysing the spatial structure in clade I haplotypes only), the results were qualitatively similar (Fig. 4b). Thus, in total, both datasets contain patterns of phylogeographic structuring that were relatively independent from divergence between clades. The correlogram including only the Finland individuals (Fig. 4c) shows an almost linear increase of *Ay* values as the geographic distances increase, indicating a clinal pattern (except for the first distance class). In contrast, *Ay* values in the combined individuals from the Oder and Elbe river basins (Fig. 4d) did not differ significantly from the random expectations in all geographic distance classes, indicating that pairs of haplotypes selected from areas in excess of 0–800 km apart are statistically independent.

Discussion

Following the human-mediated translocation of raccoon dogs from Far East to NW European Russia from 1929 to 1955, they began to invade neighbouring territories. However, in contrast to most other invading species, the understanding of the specific dynamics of biological invasions appears to be facilitated in the case of the raccoon dog by the known date of the European invasion and its geographical source, which is the NW region of the former Soviet Union. Thus, theoretical predictions regarding the general population development seemed fairly simple. Some individuals from NW European Russia would enter southern Finland and the Baltic region and would later expand westward into the wide open ecological niches. This situation would predict a probable second genetic bottleneck because the invaders would only represent a subsample of the originally translocated populations. This bottleneck would likely be followed by a fast range expansion, possibly combined with an IBD pattern of diversity, a pattern that would be consistent with other documented invasions (Sax and Brown 2000; Gaubert et al. 2009). Moreover, consistent with this pattern would also be a long-standing paradox (Allendorf and Lundquist 2003; Frankham 2005): How can a small number of initial colonists (founder population), expected to have reduced genetic diversity and thereby reduced fitness and adaptability, colonise and dominate large areas of new habitat? Although this depiction is an oversimplification of more complicated phenomena, it is a useful model that can be tested using genetic data.

The phylogenetic analysis of individual raccoon dogs across the study area permitted us to resolve patterns of genetic structure and diversity that suggested that one key to their invasion success is the co-occurrence of separate maternal lineages with divergent evolutionary histories. Of particular importance was the detection of two monophy-

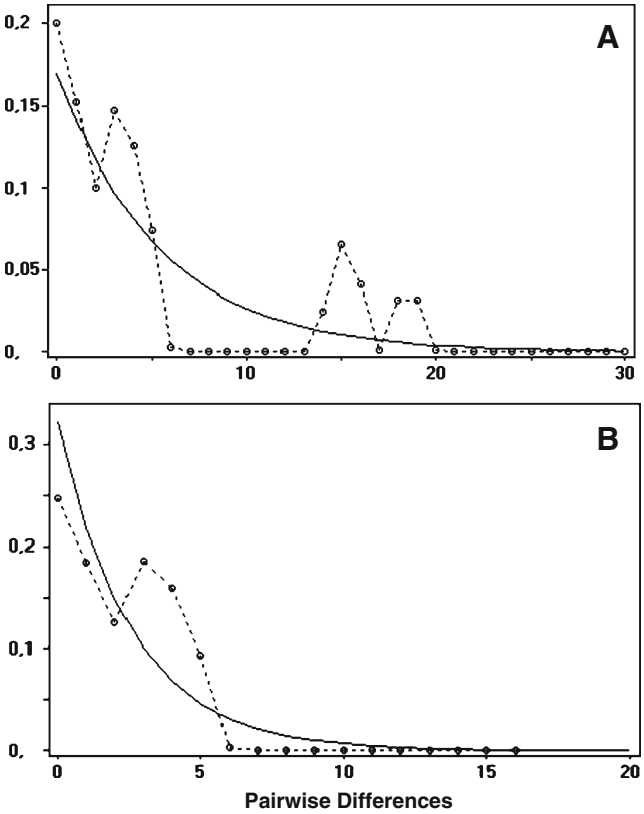

Fig. 3 Mismatch distributions for all individuals (**a**) and clade I individuals (**b**). Shown are the observed distribution (*dashed*) and the expected distribution of a population with constant size (*lines*)

letic and diagnosably distinct clades within European raccoon dogs (Fig. 2). Divergence of clades occurred between 223,300 and 773,900 years ago with a mean of 457,800 years ago. Assuming the subspecies *N. p. viverrinus* to have last shared a common ancestor with both *N. p. ussuriensis* clades, subspecies diversification within the *Nyctereutes* evolution was estimated to have occurred between 0.48 and 1.37 mya with a mean of 0.87 mya. Those ages correspond to the mid-Pleistocene epoch in continental Siberia, characterised by a transition between an extremely cold glacial period and an unusually warm interglacial period (Prokopenko et al. 2002). However, conclusions based on a molecular clock are necessarily suspect (Ayala 1999) because confidence limits around estimates of divergence are extremely large due to the Poisson mutation model of the molecular clock and potential inaccuracies in the mutation rate, in the phylogenetic tree and in the calibration dates (Hillis et al. 1996). Ideally, a species-level clock based on well-supported phylogenies could be used to increase the accuracy of the clock (Marshall 1990). Confidence in divergence date estimations becomes stronger when the latter are based on accurately dated fossils, documented biogeographical events and multiple calibration points (Marshall 1990). But to our knowledge, these approaches are not available for *N. procynoides*.

For the two primary clades, two evolutionary histories are conceivable: a single panmictic population that shows the expected deep coalescence structure (Hudson 1990) or geographical isolation followed by subsequent intermixing (Zink et al. 2006). However, if the two clades had been part of the same large source population, both should have the same genetic signature. The comparison of genetic properties showed that there were no strong evidences for clade-specific genetic signatures (Table 2). Even though mismatch distribution indices indicated at least some size expansion for the haplotypes of clade I, other statistical test indices did not corroborate this finding. Thus, the data are more consistent with a single large population of raccoon dogs in the native range which has not undergone dramatic growth.

The phylogeographic patterns of the European raccoon dogs, presenting deep genetic divergence of now sympatric lineages, are usually observed in animal groups that have experienced allopatric evolution followed by secondary contact (Avise 2000; Pitra et al. 2005). Our results agree with the idea that either a very large number of individuals (Hassan and Bonhomme 2005) or multiple introductions from several geographic areas in the native range may have been a source for the European population. Although our data did not allow to distinguish between both scenarios, it is more likely that there have been repeated, undocumented translocation events during the early period of introduction. And if so, they included individuals of already differentiated mtDNA lineages. In each case, diversification of clades must have occurred long before the recurrent introductions in Europe, most likely as a result of historical vicariance or environmentally isolating mechanisms. We propose that the current genetic population structure of European raccoon dogs followed a sequential, two-step process involving first a reduction in genetic variation due to founder effects and population bottlenecks and second an increase in genetic variation by admixture with individuals from multiple native-range sources. The lag time seen between the initial introductions in NW European Russia in 1929–1955 and the widespread establishment of raccoon dogs in southern and central Finland that began in the early 1970s is a common feature of biological invasions (Sakai et al. 2001; Lee 2002). Such delays between the initial establishment of colonists and subsequent expansion are usually explained as either the lag phase in the exponential population growth curve or the time needed to adapt to a new environment. The genetic data for raccoon dogs suggest an alternative explanation: Finland populations, established during the first expansion phase of the European invasion, contain already haplotypes of both clades, suggesting that increase in genetic variation by admixture, and not by spread of initially introduced

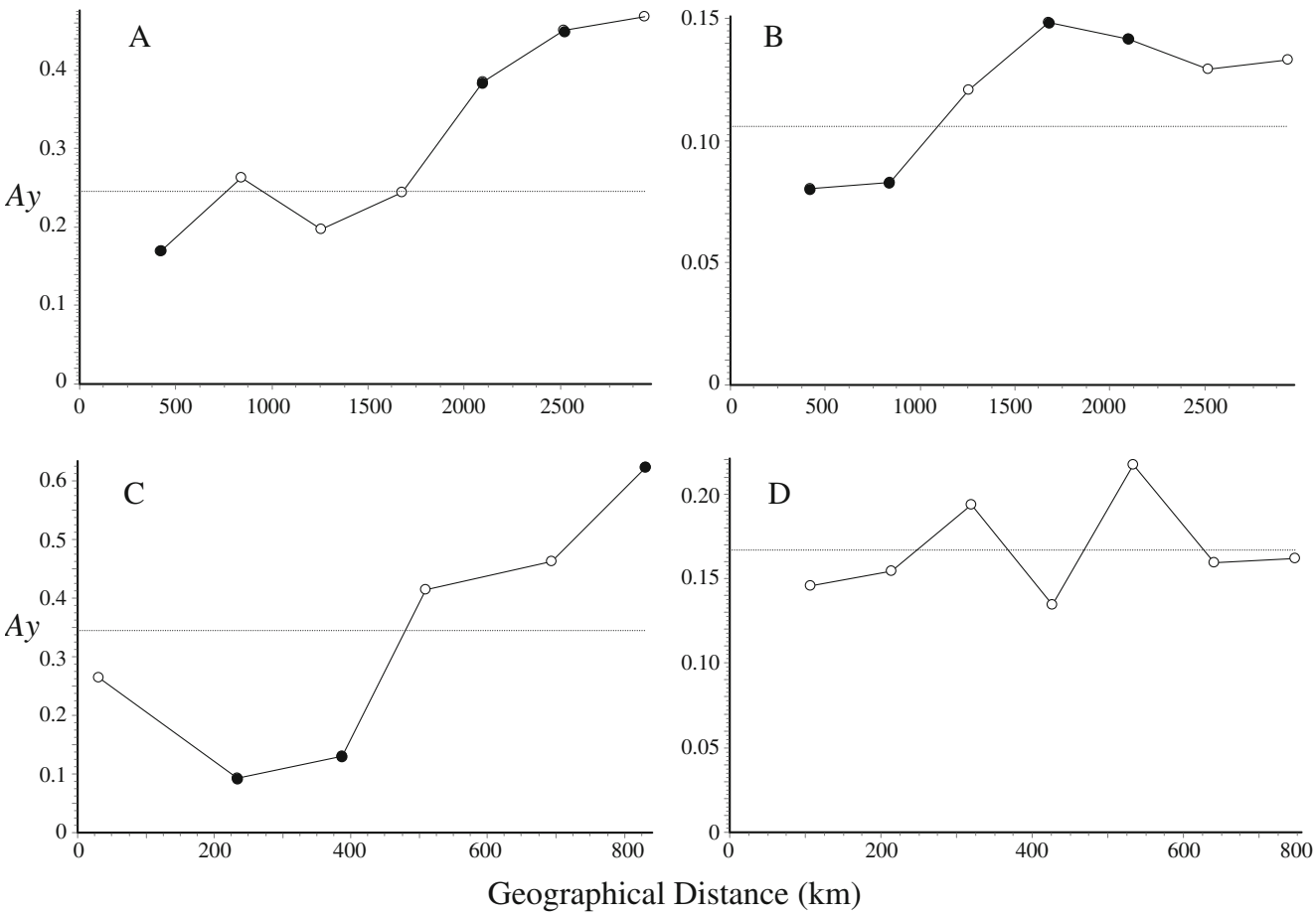

Fig. 4 Results of spatial autocorrelation analyses of raccoon dog sequences found in the the whole European dataset with (**a**) and without (**b**) clade II individuals, the drainage area of the Lake Päijänne in Finland (**c**) and the combined Oder and Elbe river basins in Germany (**d**). Analyses were performed using six to seven distinct distance classes. *Ay* quantifies the average pairwise genetic distances of haplotypes that fall within the boundaries specified for distance class *y*. Note that *Ay* takes on a value of 0 when all individuals within distance class *y* are genetically identical and takes on values of 1 when all individuals in distance class y are completely dissimilar. *Horizontal lines* indicate the average value of *Ay* for a dataset. *Black circles* represent significant ($p<0.05$) Miller's autocorrelation index (*Ay*)

individuals, may explain the range expansion. For example, the mean pairwise sequence divergence among the admixed clades I and II haplotypes in Finland was nearly 2.6 times that within clade I alone (0.98% vs. 0.38%).

The colonisation of raccoon dogs in East Germany provides a second example of this phenomenon. The first advancing raccoon dogs reached the Oder and Elbe river basins in the middle of the 1960s, but were scare for about 30 years until a rapidly increased population was noticed by rising hunting bags since about 1994 (Table 3). Today, the German raccoon dog gene pool contains seven haplotypes, five of which are shared with the Finnish gene pool, whilst two are not (H_1 and H_7; Table 1). Although the origin of the latter two haplotypes was not resolved undoubtedly in this study, the occurrence of the most abundant haplotype, H_1, in the basins of the rivers Rhine and Danube suggests an additional, probably southern invasion corridor (Lutz 1989). In each case, our results show first evidence of a

Table 3 Hunting bags of raccoon dogs in Germany from 1994 to 2006 (Deutscher Jagdschutzverband 2009)

Year	Hunted raccoon dogs
1994	407
1995	398
1996	766
1997	1.735
1998	3.249
1999	5.711
2000	7.161
2001	11.659
2002	16.087
2003	18.634
2004	23.262
2005	30.016
2006	27.512

secondary contact zone in Germany between previously geographically and genetically different source populations. Because initial introductions of raccoon dogs started in 1929 in the Ukraine, this old and well-established population may have contributed to the gene pool in central Europe (Barbu 1972). Additional studies of populations of southern Europe, especially from the Danube Delta in the Ukraine and in Romania, are warranted.

Admixture and subsequent intraspecific hybridisation of invasive populations is an extremely rapid mechanism for the increase of genetic variation and the generation of novel gene combinations on which natural selection can act. Although it is not yet clear to what extent the mitochondrial lineages revealed in this study differ in their ecology and physiology, introduction of a new genotype into a population could potentially exacerbate its existing invasive potential or initiate new such potential (Kolbe et al. 2004). Numerous studies have documented positive effects of hybridisation on invasiveness, such as faster growth, greater size and increased aggression (Ellstrand and Schierenbeck 2000; Perry et al. 2001), possibly as a result of increased genetic variance, new gene interactions, masking or unloading of deleterious recessive alleles or the transfer of favourable genes (Lee 2002). These results for human-mediated introductions contrast with natural range expansions, which do not combine geographically disparate sources. Indeed, many natural range expansions show reduction of genetic variation (Hewitt 2000).

The European invasion has increased the range and population size of raccoon dogs (Kauhala and Saeki 2004). Contrary to this observation, our data indicate that the European population has not grown much since colonisation. Both main clades separate and combined have mismatch distributions (Fig. 3) consistent with an older and more stable history. Positive Tajima's *D*, Fu's F_S and the R^2 tests also indicated that the European sample has not undergone major growth (Table 1). Relatively low levels of haplotype and nucleotide diversity in local populations (Table 1) are consistent with this view. Potentially, the growth of raccoon dog populations has been too recent to be detectable in our data. Harpending (1994) states that a population expansion that is too recent, for example at the end of the Pleistocene epoch, would not result in a smooth unimodal mismatch distribution. Additional studies of European populations are warranted and needed.

Complex patterns of post-establishment spread

Patterns of colonisation are quite complicated in *N. procynoides.* Mitochondrial diversity appears to have been preserved during the European colonisation, and no traces of founder events were detected. This translated in the scattered positions of the European samples in the phylogenetic tree (Fig. 2) rather than in their geographic grouping in a single clade with either a single or few haplotypes. The shared haplotypes of Finnish and German raccoon dogs suggests an insufficient time for divergence or the effects of stabilising selection in Europe However, Tajima's *D* test of neutrality did not show any departure from neutrality in these populations (Table 1). An AMOVA did uncover a weak structure between Finnish and German populations (13% of the variance was assigned to the difference among those regions). Our analyses of phylogeographic structure via Mantel test and spatial autocorrelation also provided evidence for population genetic structure following a pattern of IBD. Interestingly, despite the variety of factors that can contribute to phylogeographic structure over a given geographic region, both analyses consistently show that patterns of differentiation vary within the same species according to the length of time since a territory was colonised (Fig. 4). In general, the extent of genetic structure revealed by spatial autocorrelation analyses is obtained by identifying the transition point where autocorrelation coefficients switch from values that are less than average to ones that are greater than average (Clark and Richardson 2002; Diniz-Filho and Telles 2002). Although heterogeneity among *Ay* values was appreciable in all datasets, presumably due to the small sample sizes, transitions between values of *Ay* that were significantly smaller than random expectation to ones that were greater occurred in the whole European dataset (Fig. 4a, b) and in the Finnish sample (Fig. 4c). This pattern fits best to one of long-distance differentiation (Sokal and Oden 1978). In contrast, the combined group of the Oder and Elbe river basins showed non-significant values of *Ay* in all distance classes (Fig. 4d), and hence, no geographical structure was apparent. We suggest that IBD shown from mtDNA in long-established populations is likely to reflect the stepwise founding of new regional populations by females during colonisation. In contrast, no IBD was found in recently established populations, indicating that gene flow and/or recent shared ancestry homogenises gene pools among sites colonised later. IBD should be maximal at equilibrium between genetic drift and gene flow, which may take a considerable length of time to develop. Thus, the correlation between pairwise genetic divergence and pairwise geographic distance should become stronger with increasing time since colonisation. The results of our Mantel tests support this prediction. Such influence of time since colonisation was often found in empirical studies of IBD (e.g. Rafinski and Babik 2000; Castric and Bernatchez 2003).

Morphological and genetic differentiation

Our phylogenetic analysis partitioned the mtDNA variation into two main clades without any geographical structure

(Fig. 2). On the opposite, the morphological variation in the raccoon dog, as evidenced by 24 non-metric characters in the raccoon dog skull, was distributed with remarkable geographic structure (Ansorge et al. 2009). Native raccoon dogs from the Amursk region were completely separated by their skull features from the European populations. The German sample formed a separate cluster with a relatively high epigenetic distance to the Finnish–Polish group. Although there is not strict overlap between collection localities from both the morphological and genetic datasets, there is sufficient spatial concordance to make general comparisons between morphological and genetic patterns. The lack of congruence between both markers systems was not surprising, given that a meta-analysis based on 71 datasets revealed an overall weak mean correlation (r= 0.217) between molecular and quantitative measures (Reed and Frankham 2001). We suggest that spatial structure of the morphotypes can be related to hybridisation of divergent haplogroups after translocation. In addition, the discordance between the morphological and molecular patterns may have resulted from the occupation of various new habitats with varying selection regimes which would drive morphological diversification on a homogenous genetic background. Future work is required to examine the underlying genetic basis for the morphological variation observed as well as to investigate its adaptive significance.

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