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Spatio-temporal population genetics of the Danish pine marten (*Martes martes*)

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A spatio-temporal study of genetic variation in the Danish pine marten (*Martes martes*) populations from the Jutland peninsula and from the island of Sealand was performed using 11 microsatellite markers. Samples obtained from 1892 to 2003 were subdivided into historical (prior to 1970) and recent (from 1970) groups. As compared with the historical samples, there was a significant loss of genetic variation in the recent Jutland population, but not in Sealand. Effective population sizes were estimated using Bayesian-based software (TMVP). Historical effective population sizes were 5897 (90% highest probability density, HPD, limits: 1502–6849) in Jutland and 1300 (90% HPD limits: 224–5929) in Sealand, whereas recent effective population sizes were 14.7 (90% HPD limits: 10.9–23.5) in Jutland and 802 (90% HPD limits: 51.8–5510) in Sealand. Significant genetic differentiation (F_{ST}) was found between the two historical samples, between the two recent samples, and between the historical and the recent sample in Jutland; whereas the F_{ST} value between the historical and the recent sample in Sealand was not significant. The significant genetic differentiation between the historical and the recent samples indicates changes in the genetic compositions over time, and the higher F_{ST} values between the two recent samples, as compared with the two historical samples, indicates that the populations in Sealand and Jutland have drifted apart within a short time span. No deviation from Hardy–Weinberg equilibrium was found within populations, indicating no further substructuring.

ADDITIONAL KEYWORDS: ancient DNA – Bayesian statistic – effective population size – fragmentation – population decline.

INTRODUCTION

The pine marten (*Martes martes*) is distributed throughout Europe, but it has been subject to long-term decline in numbers in most regions (Mitchell-Jones *et al*., 1999). Subfossil records have shown that

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the pine marten has lived in Denmark for the last 9500 years, and it is believed to have invaded the country 11 000 years ago when the area was recolonized by forest after the latest ice age (Aaris-Sørensen, 2007; Madsen *et al*., 2007). At present it is listed as endangered on the national Red List (Stoltze & Pihl, 1997). It is a habitat specialist confined to mature deciduous and coniferous forests (Domingo-Roura, 2002), has a limited dispersal ability compared with other mustelids (Kyle, Davis & Strobeck, 2000), and a slow reproduction rate, rendering it particularly vulnerable to habitat changes (Bright, 2000). Over the last centuries, the landscape in Denmark has undergone substantial changes. Today, it consists of large areas of open, cultivated land with a few, widely dispersed fragments of natural habitat (see Caspersen, 2001). Habitat destruction and deterioration resulting from fragmentation is supposed to be the main cause of the decline of the pine marten (Degn & Jensen, 1977).

Population declines have been observed in several mustelids in Denmark from the early 1960s: e.g. polecat (*Mustela putorius*) (Møller *et al*., 2004), Eurasian otter (*Lutra lutra*) (Pertoldi *et al*., 1997, 2001a, b), Eurasian badger (*Meles meles*) (Pertoldi *et al*., 2003, 2006), weasel (*Mustela erminea*), and stoat (*Mustela nivalis*) (Pertoldi *et al*., 2006). The reasons for this general decline seem to be associated mainly with the intensification of agricultural practices, and the consequent habitat fragmentation, which increased from the beginning of the 1960s (Caspersen, 2001; Pertoldi *et al*., 2003).

Effects of habitat fragmentation

Habitat fragmentation has affected numerous species by interrupting or reducing gene flow between populations (Pope, 1996; Galbusera *et al*., 2000), and often causing a decline in the effective population size (N_e) (Frankham, 1995). Large populations of naturally outbreeding species usually have extensive genetic diversity, but this is often reduced in populations and species of conservation concern (Frankham, 1995). In particular, carnivores appear to have low genetic variability relative to other mammals (Merola, 1994; Pertoldi *et al*., 2001a, 2005; Randi *et al*., 2003). Low genetic variability may lead to increased inbreeding depression, but this depends on whether the low variability is of recent origin or whether it is the result of demographic fluctuations. Information from historic samples would improve estimates of the loss (or maintenance) of genetic diversity over time.

Several recent studies have included historical samples from museum collections, or other types of archived samples representing pre-fragmentation or pre-decline time periods, which were compared with data from present populations. In some cases, genetic diversity had decreased as a result of a population crash (Westemeier *et al*., 1998; Glenn, Stephan & Braun, 1999; Weber *et al*., 2000; Bellinger *et al*., 2003; Flagstad *et al*., 2003), whereas low diversity preceded the more recent population decline in other cases (Pertoldi *et al*., 2001a; Paxinos *et al*., 2002; Hadly *et al*., 2003).

Recent reduction of N_e can generate large genetic differentiation among populations, and may drastically reduce the genetic variability within populations in a short time (Hedrick, 1999). We hypothesized that the documented decline in population size, together with habitat fragmentation, would be reflected in (1) genetic differentiation between geographical regions, and (2) changes in genetic variability over time within geographical regions. The spatial differentiation hypothesis was tested using samples from two isolated geographical regions: the Jutland peninsula (J) and the island of Sealand (S). The hypothesis of temporal loss of genetic variability was tested by comparing historical and recent samples from each geographical region.

MATERIAL AND METHODS

Origin of the samples

Samples from 103 Danish pine marten were collected between 1892 and 2003 from two geographical regions (J and S) separated by the sea, which we assumed to be a geographical barrier (Fig. 1). The material was further subdivided into historical and recent samples. The two historical samples were Jutland prior to 1970 (J < 1970), $n = 18$, and Sealand prior to 1970 (S < 1970), $n = 28$. The two recent samples were Jutland 1970–2003 (J > 1970), $n = 38$, and Sealand 1970–2003 (S > 1970), $n = 19$. The recent and historical samples were stored at the collections of the Museum of Natural History, Aarhus, the Zoological Museum, Copenhagen, and at the National Environmental Research Institute, University of Aarhus, Kalø, and originated from either road kills, animals caught in traps, or animals shot by hunters.

DNA extraction

From the recent samples, DNA was extracted from muscle, kidney, or hair samples using standard chloroform/CTAB extraction (Murray & Thompson, 1980). From the historical samples, DNA was extracted from hair samples using standard chloroform/CTAB extraction, and from teeth using microconcentrators (Nielsen, Hansen & Loeschcke, 1999; Pertoldi *et al*., 2001a). Material from teeth was obtained by removing a canine tooth and drilling out the root using a 2-mm drill. Approximately 0.15 g of

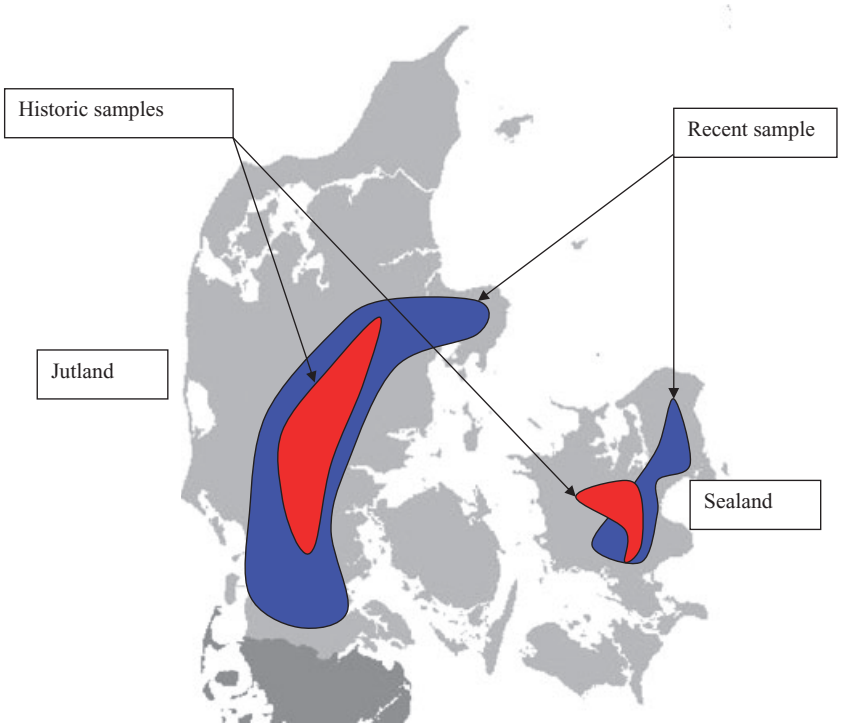

Figure1. Map of Denmark with the locations of both the historical and recent pine marten samples.

tooth and tooth root was collected and used for DNA extraction following the procedure of Pertoldi *et al*. (2006). The drill was sterilized by heating following each collection of tooth root in order to avoid cross-contamination. Extractions of DNA from recent tissue samples were not conducted in the laboratory during the same time period as work on historical samples, and polymerase chain reaction (PCR) reagents were exposed to UV radiation in a UV cross-linker in order to degrade possible contaminating DNA (see Pertoldi *et al*., 2001a).

Microsatellite analysis

The 11 dinucleotide microsatellite primer sets were originally developed by Bijlsma *et al*. (2000) for *M. meles* (Mel 1 and Mel 6), Davis & Strobeck (1998) for *Martes americana* (Ma 1 and Ma 4), Domingo-Roura (2002) for *Mustela vison* (Mvi 39 and Mvi 57) and O'Connell, Wright & Farid (1996) (Mvi 72), Fleming, Ostrander & Cook (1999) for *M erminea* (Mer 22, Mer 41 and Mer 95), and Dallas & Piertney (1998) for *L. lutra* (Lut 615).

Recent samples were run for 40 cycles of PCR and historical samples were run for 50 cycles. PCR reactions were run in either 10-μL or 5-μL volumes, with either 1-μL or 0.5-μL of DNA, respectively, from each sample. The reaction (10-μL volume) also included: 1.0 μL 10*buffer, 1.6 μL deoxyribonucleotide triphosphate (dNTP)-mix, 5 pmol forward primer, 5 pmol backward primer, 0.1 μL taq enzyme, and H_2O until the volume was 10 μL in all. The above quantities were halved for the 5-μL volume. PCR was run on PCR Express from Hybaid and PTC-200 (a Peltier Thermal Cycler) from MJ Research. The PCR was run at 94 °C for 3 min to denature the samples, and each cycle consisted of strand separation at 94 °C for 30 s, annealing temperature (50– °C) for 40 s, and elongation at 72 °C for 1 min. Each cycle ended at 72 °C for 7 min. The optimal annealing temperatures were determined using a temperature gradient from 50 °C to 60 °C, followed by inspection of products on an agarose gel.

Most of the historical samples originating from teeth did not provide sufficient quantities of DNA using the usual PCR protocol. Therefore, the PCR was preceded by amplification without primers (25 cycles of 92 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min) (Golenberg, Bickel & Weihs, 1996). The PCR products were run on a 24% acrylamide gel on ALF EXPRESS and ALF EXPRESS II, from Amersham Pharmacia Biotech, following the manufacturer's recommendations. The peaks were scored using ALF WIN FRAGMENT ANALYSER 1.0 using a 2% detection level.

Previous studies have shown that allelic drop-out (amplification of just one of two alleles) may occur in analyses of degraded DNA obtained from old teeth and bones (Woodroffe, Macdonald & da Silva, 1995;

Zierdt, Hummel & Herrmann, 1996), presumably resulting from a scarcity of intact DNA templates (Hummel & Herrmann, 1995). In addition, the primers used in this investigation were not designed for the pine marten, which could produce an increase in apparent homozygous genotypes because of null alleles. To verify the reliability of our results, screening was performed twice for each sample, and a second tooth was tested for all apparent homozygotes, to check the reliability of the scoring. Alleles were scored without prior knowledge of the sampled populations, and no discrepancies between the scorings were found. We conclude that allelic drop-out did not significantly affect our results. Furthermore, the observed Hardy–Weinberg equilibrium (HWE) within populations for both the historical and the recent samples (see the Results section) allowed us to exclude the presence of null alleles.

Statistical analyses

All microsatellite loci and samples, considered separately or pooled, were tested for deviations from HWE using GENETIX ver. 4.04 (Belkhir, 2000; http://www.univ-montp2.fr/~genetix/genetix/genetix.htm). Significance levels were adjusted using the sequential Bonferroni correction for multiple comparisons (Rice, 1989).

Genetix was also used for the estimation of the pairwise F_{ST}s and their associated P values. The F_{ST} values were estimated between the two recent samples, between the two historical samples, and between the historical and recent samples within the same geographical region.

Expected heterozygosity (H_e) and allelic richness (AR) were estimated using FSTAT 2.9.3.2 (Goudet, 2001; http://www2.unil.ch/popgen/softwares/fstat. htm). The arcsine-transformed values of H_e and mean AR within samples were tested for differences between the recent sample and the historical sample within each geographical region.

Effective population size

As the population sizes of both the Sealand and Jutland populations apparently have declined over the last century, we used the TMVP package (Beaumont, 2003) to estimate N_e for the recent (post-1970) and historical (pre-1970) periods. The historical samples were subdivided according to the date of collection into ten subsamples for Sealand and six for Jutland, and for each subsample we estimated the time in generations to the most recent sample, assuming a generation interval of three years. For each of the two populations, tmvp2p was run for five replicates of 100 000 updates, with a maximum population size of 7000 (i.e. a rectangular prior of 0, 7000) for both ancestral (N_A) and recent (N_0) effective population sizes. Parameters were set with MAXIT as 600 (Jutland) and 700 (Sealand), and size equal to 0.1 for both regions. Every 20th update after the first 100 was included in the trace. For the final analysis of the five replicates, the second half of the output from each was combined (a total of 12 500 updates), and the posterior distributions of N_A and N_0 were evaluated.

RESULTS

Genetic variation

Levels of genetic variation estimated as H_e and AR were high (Table 1), both in historical (J < 1970, $H_e = 0.79$, $AR = 5.19$; S < 1970, $H_e = 0.74$, $AR = 4.86$) and recent samples (J > 1970, $H_e = 0.67$, $AR = 4.03$; S > 1970, $H_e = 0.72$, $AR = 4.39$). The reductions in AR and H_e in the recent J sample, as compared with the historical J sample, were both significant (AR, $P = 0.03$; H_e, $P = 0.02$). However, neither was significant for the S samples.

Hardy–Weinberg equilibrium

There were no overall deviations from HWE within any of the four samples. For individual loci, there were 13 significant deviations from HWE (eight resulting from heterozygote deficiency and five from heterozygote excess), but none were significant after Bonferroni correction.

Genetic differentiation

The F_{ST} values between the historical samples and between the recent samples were significant (historical, J < 1970 vs S < 1970, $F_{ST} = 0.044$, 95% consistency index, CI = 0.018–0.081; recent, J > 1970 vs S > 1970, $F_{ST} = 0.097$; 95% CI = 0.051–0.142). Pairwise F_{ST} for the historical and recent samples were significant for the J region (J < 1970 vs J > 1970, $F_{ST} = 0.083$, 95% CI = 0.043–0.122), but not for the S region (S < 1970 vs S > 1970, $F_{ST} = 0.002$, $P > 0.05$).

Effective population size

The posterior distributions of N_A and N_0 (Fig. 2A) show very strong evidence of population decline of the Jutland population, with a joint mode from the density estimation of $N_A = 5897$ and $N_0 = 14.7$. The modes and 90% HPD limits for the marginal estimates are 5963 individuals (1502–6849) and 15.1 individuals (10.9–23.5) for N_A and N_0, respectively. The Bayes factor in favour of decline is 9.43 (where > 2 is judged as significant).

Table1. Summary allelic richness (*AR*) and expected heterozygosity (H_e) per locus, and population and mean *AR* and H_e per population. The four samples were constituted by two historical samples, Jutland prior to 1970 (J < 1970) and Sealand prior to 1970 (S < 1970), and two recent samples, Jutland 1970–2003 (J > 1970) and Sealand 1970–2003 (S > 1970)

	Historic		Recent		Historic		Recent	
Locus/population	$n = 18$ *AR*	(J < 1970) H_e	$n = 38$ *AR*	(J > 1970) H_e	$n = 28$ *AR*	(S < 1970) H_e	$n = 19$ *AR*	(S > 1970) H_e
Mer41	4.94	0.77	3.88	0.73	4.33	0.77	3.81	0.73
Mel1	5.32	0.82	4.59	0.77	5.62	0.83	5.24	0.82
Mel6	3.71	0.65	1.92	0.21	3.11	0.44	3.23	0.39
Mer95	6.09	0.85	4.87	0.75	7.29	0.88	4.55	0.71
Mvi39	8.11	0.93	4.69	0.72	6.93	0.87	7.03	0.90
Lut615	6.98	0.89	4.09	0.67	4.95	0.79	4.86	0.79
Mer22	4.00	0.76	3.08	0.58	5.00	0.85	3.99	0.78
Mvi57	4.66	0.73	4.70	0.79	3.01	0.56	4.25	0.70
Ma4	5.08	0.77	4.98	0.78	3.79	0.71	4.10	0.74
Ma1	4.27	0.77	3.92	0.71	4.33	0.69	4.19	0.70
Mvi72	3.94	0.76	3.65	0.67	5.05	0.80	3.00	0.68
Overall	5.19	0.79	4.03	0.67	4.86	0.74	4.39	0.72

The Sealand population, in contrast with that in Jutland, shows no significant evidence of decline. The posterior distributions of N_A and N_0 (Fig. 2B) show a joint mode of $N_A = 1300$ and $N_0 = 802$, but both this mode and the line of equal population size are within the 10% HPD limits. The Bayes factor is not significant. The marginal modes and 90% HPD limits are 1057 (224–5929) for N_A and 505 (51.8–5510) for N_0, showing substantial overlap.

DISCUSSION

Genetic variability and divergence in mustelids

Although the level of genetic variability found in the recent and historical samples was high, compared with the variability found in other studies on pine marten and other species belonging to the Mustelidae family, genetic divergence between the two regions was relatively low. Studying pine marten populations across Europe, Kyle, Davison & Strobeck (2003) found microsatellite H_e values in the range of 0.34–0.66 and F_{ST} values in the range 0.016–0.330. For fisher marten (*Martes pennanti*) in North America, Kyle, Robitaille & Strobeck (2001) found the average microsatellite $H_e = 0.62$, whereas F_{ST} ranged from 0.028 to 0.261. A survey on wolverine (*Gulo gulo*) in Scandinavia using microsatellites showed low genetic variation (0.345–0.393) and F_{ST} values ranging from 0.023 to 0.142 (Walker *et al*., 2001). The lower genetic divergence in our study may reflect the small geographical scale sampled, as compared with other studies. It may also in part be the result of the high level of heterozygosity, which leads to a lower maximum F_{ST} value (Hedrick, 1999).

The Danish pine marten populations showed both spatial and temporal genetic divergence, with the significant differentiation between geographical regions increasing from the historical to the recent samples. However, this increasing differentiation was primarily caused by genetic changes in the Jutland population, as the F_{ST} for the historic/recent samples from Sealand was not significant. Expected heterozygosity and allelic richness show the same pattern (Table 1), with a significant decrease in both only in the Jutland population. Given the extent of the apparent habitat fragmentation and the dramatic reduction in N_e in Jutland, genetic structuring might have been expected in this population, as would be evidenced by deviations from HWE. As none were found, the limited dispersal ability of the pine marten (Kyle *et al*., 2000) is apparently sufficient to maintain a panmictic population.

Temporal changes in genetic variability for Danish pine marten

The use of historical samples has allowed us to assess patterns of genetic composition of pine marten populations over a longer time scale than would otherwise have been possible, and thereby to detect an increase of the genetic divergence between the two recent samples, as compared with the two historical samples, providing information on the temporal stability of genetic differentiation among populations. The Bayesian approach allowed us to confirm the

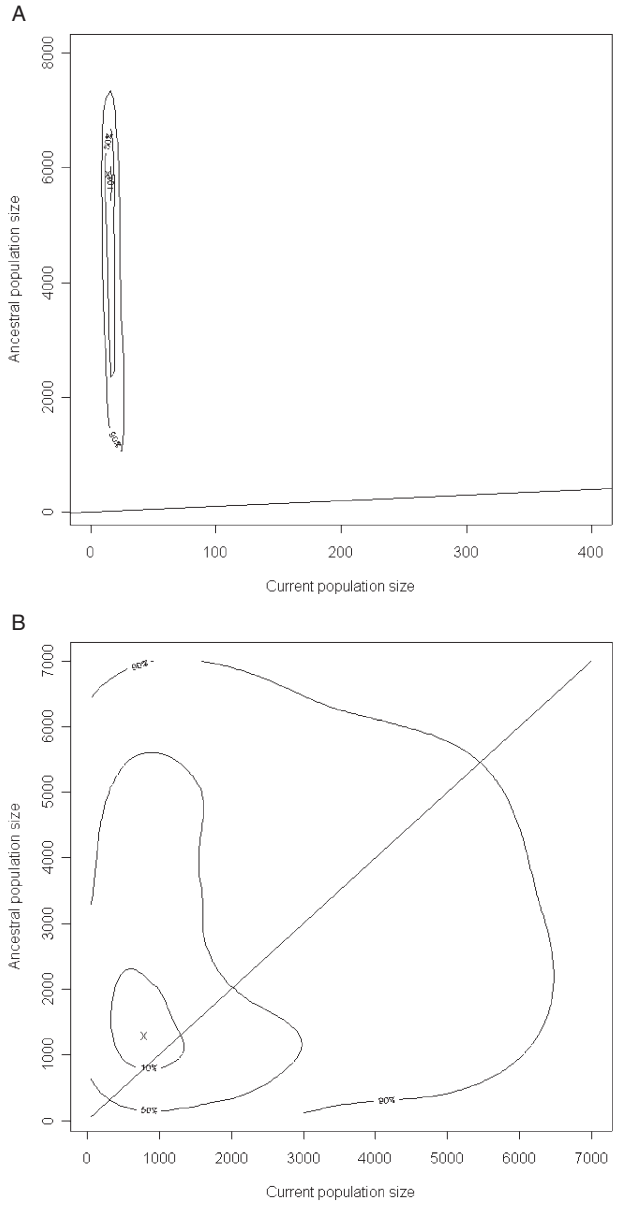

Figure2. Posterior distribution of ancestral (N_A) and recent (N_0) effective population sizes for Jutland (A) and for Sealand (B). The contour levels correspond to 10, 50, and 90% highest probability density (HPD) limits, the straight line indicates $N_A = N_0$, and the bivariate mode is marked by a cross.

drastic population reduction observed in Jutland and the demographic population close to status quo of the Sealand population. The Jutland population was larger than that of Sealand prior to 1970. But the subsequent population decline was much more drastic in Jutland, where the recent N_e is only 0.2% of the historical N_e, as compared with 62% in Sealand. This difference is likely to have resulted from the more intensive agricultural development in Jutland, as compared with Sealand, over the past 30–40 years.

Pine marten in the future landscape

From a conservation point of view, only the analysis of the population previous to the perturbation would unambiguously reveal the basis against which to evaluate the current genetic status of the species. The pine marten has declined considerably in numbers, but the present loss of genetic variation for microsatellite loci is not a cause for great concern, as molecular markers cannot identify the likelihood of a loss of genetic variance in traits of ecological significance, as the correlation between molecular diversity (e.g. heterozygosity) and quantitative genetic variation (e.g. heritability) is weak, and becomes even weaker in expanding or declining populations (Gilligan, Briscoe & Frankham, 2005). However, the populations are considerably diverged from each other, showing differentiation between the geographical regions.

The high genetic variability detected could be the result of metapopulation dynamics, where a few individuals carrying rare alleles occupy new empty patches. These rare alleles would have had the chance to increase in frequency, and consequently increase H_e, whereas in a large population the opposite occurs, as the rare alleles would have barely increased in frequency. Furthermore, we cannot exclude the possibility that, given the relatively fast demographic changes in the Jutland population in the last 30–40 years, the populations have not yet reached the mutation–drift equilibrium. In this case, the level of genetic variability detected would still be decreasing and would continue to decrease until it reached the mutation–drift equilibrium. This equilibrium point should be much lower than the equilibrium point expected for the Sealand population, given the considerably larger N_e of the present Sealand population.

Despite the fact that the present populations do not seem to be genetically depauperate, we cannot exclude the possibility that the Jutland population has a genetic load caused by the relatively fast loss of genetic variability. In fact, it is not only the current level and structure of genetic diversity that is important to the continued existence of wild populations. The distribution of genetic diversity with respect to the recent past, and the rate of change, must also be carefully investigated, as the purging of deleterious alleles only works when inbreeding occurs gradually and over several generations (Reed *et al*., 2003). Hence, if inbreeding is sudden and extreme, N_e is reduced and drift becomes predominant relative to selection, resulting in more random fixations, even for recessive deleterious alleles (Day, Bryant & Meffert,

2003). Sealand became isolated as an island 7500–8000 years ago (Aaris-Sørensen, 2007), and since then the inner Danish waters have formed an absolute barrier between the two populations of pine marten of Jutland and Sealand. An increased connectivenes between the Jutland and the Sealand populations, which could be obtained by a translocation strategy, should be made with an asymmetric gene flow, with the Sealand population as the source and Jutland as the sink population. The reverse may be quite dangerous, as a genetic load could be introduced into the Sealand population.

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