Estimating population differentiation between isolated root vole (*Microtus oeconomus*) populations in the Netherlands using geometric morphometrics

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Abstract: We investigated morphological differentiation in threatened populations of the root vole subspecies *Microtus oeconomus arenicola*, measured by using geometric morphometrics on skulls recovered from owl pellets. Using populations from Finland as a reference, we compared measures of morphological differentiation to levels of genetic differentiation reported in literature for the same populations. We found that the degree of morphometric population differentiation was generally lower than the degree of genetic differentiation, yet it revealed broadly similar patterns of geographic isolation. This suggests that skull shape is conserved in isolated root vole populations, and that geometric morphometric measurements from skeletal parts recovered from owl pellets may provide a cost-effective method to monitor population subdivision.

Keywords: conservation, population subdivision, habitat fragmentation, skull, root vole.

Introduction

Habitat loss and fragmentation are well known factors to threaten persistence of animal species through isolation of local populations (Hartl & Clark 1989). A high degree of isolation is very generally accompanied by a high degree of inbreeding, which decreases fitness through increased homozygosity and susceptibility to diseases (Frankham 1996). These factors slow the recovery of populations after major disturbances, rendering local populations more vulnerable to demographic fluctuations, and eventually (in combination with reduced recolonisation) more susceptible to extinction (Frankham et al. 2002).

Conservation efforts often study population genetic structure of endangered species solely in order to measure the degree of isolation of local populations as a proxy for various other threats. A popular measure to quantify the genetic variability of populations is:

$$F_{ST} = \frac{V_b}{V_b + V_w}$$

(e.g. Weir & Cockerham 1984), where V is genetic variation between (V_b) and within (V_w) populations. As is easily understood from the above equation, $0 < F_{ST} < 1$. F_{ST} does not measure the total genetic variation in the population ($V_w + V_b$), but the variation that is found between populations, or rather between individuals within populations. More precisely, if F_{ST} is calculated from genetic markers that are

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not under selection (e.g. microsatellites) and if rates of mutation are similar in different populations (Nagylaki 1998) then F_{ST} is governed by the effects of migration and random genetic drift (Kimura 1983, Hartl & Clark 1989), and measures the degree of population differentiation resulting from drift and gene flow (Lande 1992). Hence, F_{ST} provides information about the degree of population isolation: if local populations are isolated and the genetic variation within populations is low, most genetic variation will exist between populations, resulting in a high value of F_{ST}. On the other hand, if there is extensive gene flow between local populations, much genetic variation is expected between individuals of the same local population, and little differentiation between populations, so that F_{ST} will be close to 0. From the above, it will be clear that population genetic analysis of endangered species can provide a wealth of information for conservation efforts, however, it should be kept in mind that the lack of genetic variation rarely threatens populations by itself, except through direct negative effects on survival and fecundity under extreme levels of inbreeding (Lande 1998, but see Spielman et al. 2004). The most general way in which decreased variation of local populations increases local extinction risk is probably a reduction in the capacity to adapt to changes in the environment: isolation decreases the genetic variation within a local population, and renders potentially useful genetic variants present in other populations inaccessible, thereby reducing the local population's capacity for adaptive evolution (Hartl & Clark 1989). Therefore, it is useful to infer not only the degree of genetic subdivision of a population, but also the degree of local adaptation.

The degree to which populations are adapted to their local environment can be inferred by comparing phenotypic (e.g. morphological) variation with genetic variation. In a similar fashion as explained for genetic variation above, phenotypic variation can be partitioned into within- and between-population components. Specifically, we can calculate

$$P_{ST} = \frac{P_b}{2 * P_W + P_B}$$

where P_B is phenotypic variation between and P_W phenotypic variation within populations. Again, low levels of P_{ST} indicate that most phenotypic variation is found within populations, which implies little phenotypic differentiation of populations. If P_{ST} is high, on the other hand, populations are phenotypically distinct but foster little variation within them. It was shown (Spitze 1993) that when neglecting phenotypic differences due to different environments (for diploid species, assuming purely additive gene action and no linkage disequilibrium), P_{ST} is analogous to F_{ST}. That is, P_{ST} is the value of F_{ST} that would be obtained if F_{ST} were calculated from the genes that determine the phenotype (Wright 1951, Lynch & Spitze 1994, Latta 1998).

The analogy between P_{ST} and F_{ST} facilitates comparison of the variation in neutral (microsatellite) markers (F_{ST}) and that in metric traits (P_{ST}): A difference between the two values can tell us something about the direction of natural selection (McKay & Latta 2002). Typically, for divergent selection, where two populations become adapted to different environments, the degree of phenotypic differentiation between populations exceeds the degree of differentiation at neutral loci, so that $P_{ST} > F_{ST}$. Conversely, if the direction of selection is towards equal phenotypes in several populations (convergent selection), phenotypic variation between populations is smaller than genetic variation between the same populations, so that $P_{ST} < F_{ST}$. If $P_{ST} \approx F_{ST}$, the effects of genetic drift and selection are indistinguishable (Merilä & Crnokrak 2001).

Comparisons of the genetic and phenotypic structure of populations can be helpful to determine the risk for isolated populations to become vulnerable to stochastic events. This is of great interest for conservation biology, where

the goal is to preserve variation within populations and to ensure connectivity between sub-populations. An example of a population threatened by fragmentation due to human impact is the root vole (*Microtus oeconomus*). The Dutch subspecies, *M. oeconomus arenicola*, is the Netherlands' only endemic mammal subspecies and is endangered: its occurrence has been threatened by habitat fragmentation and loss during the last century. Recently, human activities have enabled other vole species (common vole, *M. arvalis* and field vole, *M. agrestis*) to colonise areas that were previously the exclusive domain of *M. oeconomus*. In these places, those invading species outcompete *M. oeconomus*, mainly in the drier parts of its habitat. This process further reduces the range of the root vole in the Netherlands (La Haye & Drees 2004). The population genetic structure of Dutch root vole populations has been studied using allozymes and microsatellite markers. While allozyme studies indicated low levels of genetic variation in local populations (Leijs et al. 1999), analyses using microsatellite markers showed that genetic differentiation is as large between regions within the Netherlands as it is between Dutch and Scandinavian populations (van de Zande et al. 2000). But, allozymes are variant forms of an enzyme that are coded by different alleles at the same locus, and may therefore reveal only genetic variation resulting from structural changes in enzymes. Thus, allozymes are more prone to selection bias than microsatellites and have a lower resolution in measuring genetic diversity. This suggests that populations of *M. oeconomus arenicola* experience substantial genetic isolation.

In this study we estimate morphological variation in root vole populations (P_{ST}) and compare it to literature reports of genetic variation (F_{ST}) from the same populations to infer selection regimes. We measure morphological variation from skulls found in regurgitated pellets of the barn owl (*Tyto alba*) and long-eared owl (*Asio otus*). Using this non-invasive sampling method we avoid removing individuals from the population. We quantify skull morphology using geometric morphometrics, which is particularly sensitive to small morphological differences, and has earlier been applied successfully to show differences between root vole populations in Hungary (Ràcz et al. 2005).

Methods

Study species

The root vole has an almost circumpolar geographic range from northern Scandinavia eastward to Siberia, into Alaska and Canada. The main population stays above 50° north, but several isolated relict populations are remnants of a more southern postglacial distribution. In Europe, such relict populations can be found in Mid-Norway, Finland, Austria, Hungary, Slovakia and the Netherlands. Because of its endangered state, the Dutch root vole subspecies *M. o. arenicola* is included in the European Community Habitats Directive (97/62/EC) as a priority species; it is also classified as Critically Endangered (CR) by the IUCN (Gippoliti 2002 in: IUCN 2006) and it is on the Dutch 'Red List' for endangered mammals (Thissen et al. 2009).

Sampling

Root vole skulls from Dutch vole populations were obtained from barn owl and long-eared owl pellets. Home ranges of the owls are up to 5 km^2 in size (Arlettaz et al. 2010), so that the scattered occurrence of root vole populations renders it unlikely that pellets produced by an individual owl contain rodent samples from more than one region. For reference, we also used specimens from Finnish root vole populations, which were obtained from the zoological museum of the University of Oulu, Finland. These specimens had been collected by trapping at various locations.

The Dutch samples came from five regions; four of the five regional clusters described in the *Beschermingsplan Noordse Woelmuis* (Pro-

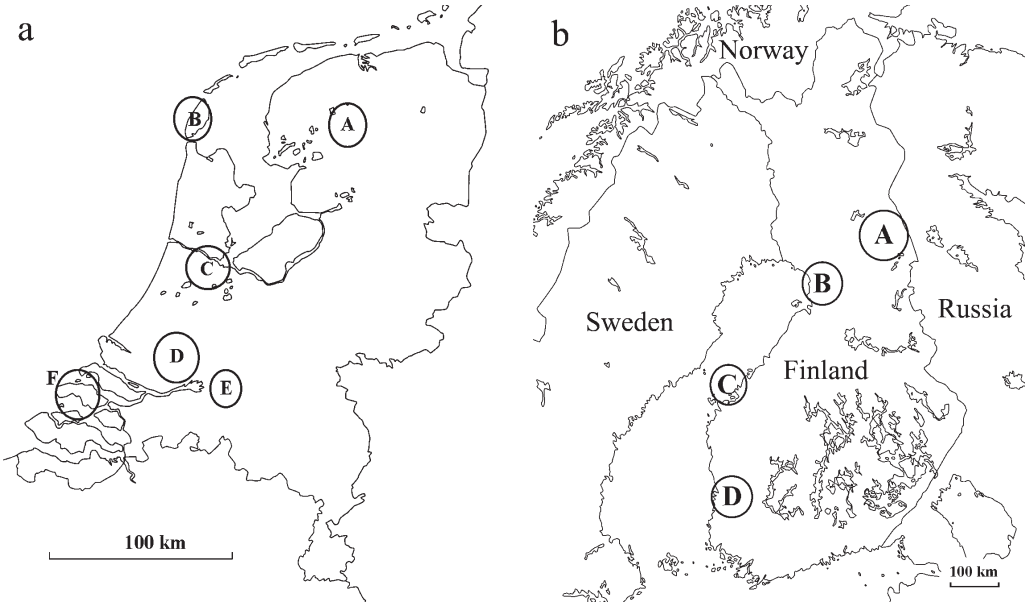

Figure 1. Maps showing sampling locations in the Netherlands (a) and Finland (b). Letters in the circles of figure 1a correspond with regional clusters of the Netherlands: A=Fryslân; B=Texel; C= Zeeland; D= Zuid-Holland; E= Biesbosch. Letters in the circles of figure 1b correspond with sampling locations in Finland: A=Kuusamo, B=Li, C=Tankari, D=Ahlainen.

tection plan Root Vole, La Haye & Drees 2004). The fifth region in this study is the Biesbosch area, a swamp which represents a habitat distinctly different from the neighbouring regions of Zeeland and Zuid-Holland (figure 1a). For Finland, populations were not combined into regions, since they are situated sufficiently far from each other to be all considered representative of separate regions (figure 1b). Sample sizes *n* were as follows: Fryslân: 60, Texel: 11, Zeeland: 181, Zuid-Holland: 56, Noord-Holland: 2, Biesbosch: 55, Kuusamo: 8, Li: 22, Tankari: 5, Ahlainen: 5. A table with exact locations and populations sampled is available from the authors upon request.

Geometric morphometrics

We used geometric morphometrics to quantify skull shape. Geometric morphometrics analyses the geometric configuration of a set of corresponding points on each specimen under study. These points, often placed at diagnostic features, such as the tip of the skull, or bone fissures, are termed landmarks, a term borrowed from craniometry and previously from topographic surveying. The analyses of this data use mathematical definitions of shape. The shape incorporates all features of the landmarks, except for *size, position* and *orientation*. A so-called Procrustes transformation can remove these factors from the landmark configuration, making the remaining descriptors suitable for standard multivariate analyses. The removal of size is achieved by scaling all samples to the same centroid size (the square root of the sum of landmark distances from the centroid point). Subsequently, centroids of all samples are superimposed. Finally, all samples are rotated for an optimal fit, in order to minimise distances between corresponding landmarks between individuals. (For statistical background of the process see Rohlf & Slice (1990) and Bookstein (1991, 1996)). The remaining variation in landmark

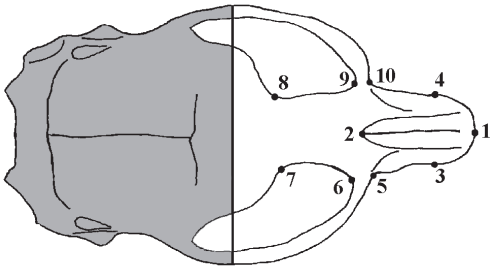

Figure 2. Landmarks used for morphometric analysis, on a skull of *M. oeconomus*. The grey area at the back half of the skull is usually broken off, and therefore no landmarks could be selected in that area (figure adapted from Ràcz et al. 2005).

coordinates is variation in shape and can be used as input for standard multivariate statistics (Klingenberg & McIntyre 1998).

Geometric morphometrics possess two important advantages over traditional methods. The first is its ability to represent results graphically, which allows easy interpretation in relation to the object under study. Second is its remarkable statistical power, enabling detection of even very small phenotypic differences (Klingenberg et al. 2002).

Landmarks and selection of skulls

We used eight of the landmarks used by Ràcz et al. (2005) in their study of the root vole, plus two extra, all located on the front half of the skull. Ràcz et al.'s landmarks located on the braincase could not be used, since this part is usually fractured and missing in owl pellets. The complete representation of landmark locations is given in figure 2.

For the selection of landmarks, a trade-off between as many landmarks as possible and as many samples as possible has to be made. Reduction in either of the two presents unwanted difficulties in concurrent statistical analyses, as discussed extensively by Adams et al. (2004). Thus, it was decided to concentrate on landmarks on the frontal part of the cranium, as most skulls, including the relatively damaged ones, were intact in this part. Those skulls that were damaged in such a manner that not all landmarks were present, had to be removed from analysis, since for a Generalized Procrustes Analysis (GPA) it is necessary that all samples have equal numbers of landmarks. To date, there is no satisfactory solution to deal with this problem (Adams et al. 2004).

Preparation for analysis

Skulls from pellets were cleaned with a brush, hair and mud were removed with a pair of tweezers. Each skull was assigned a unique identification code. Each skull was photographed from a dorsal view with a tripod mounted Olympus E-500 digital camera. Included on each photograph was a fixed distance line as well as the unique identification code, to prevent accidental mixing-up of images. The digital images were then randomised using the program TpsUtil 1.34 (Rohlf 2005) before marking landmarks.

Ten landmarks were marked on each skull using tpsDig version 2.05 software (Rohlf 2006). To assess the accuracy of the measurements, VB measured all skulls twice in random order and from those measurements we calculated repeatabilities. For both series of measurements, all X and Y-coordinates of the ten landmarks were added up, to obtain one number per individual skull measured. Following Lessels & Boag (1987) repeatability was calculated based on a one-way ANOVA from this data with identity as factor and the two measures as response. Measurements proved to be very accurate with a repeatability of 0.9998 (se $= 4.3*10^{-5}$, $F_{1,364} = 8030.5$, $P<0.0001$). Further analyses were performed based on the averaged values form the two measurements.

Levels of comparison

The populations were compared at the level of the country, at the level of the region within

countries, and for the Dutch populations also at the population level. The data was entered into the statistical software PAST version 1.42 (Hammer et al. 2001), where the landmark data was transformed using Procrustes analysis. With this data a Shape Principal Components Analysis was performed, to identify the principal components (PC) that best described the variation in skull shape. From inspection of plots of magnitude, direction, and size of principle components, it was decided that only the first two principle components reflected systematic shape variation.

Subsequently, a MANOVA on the first two PCs was performed to identify differences in skull shape and finally, Hotelling's T^2 test was used to identify which pairs of populations were significantly differentiated in skull shape. In the computer program MATLAB a dendrogram based on the MANOVA was made, to visualise the differences of the different populations, using Mahalanobis distances between group means (A Mahalanobis distance tree is roughly equivalent to a phylogenetic tree, in that it expresses the amount of phenotypic variation between populations as distances between them. This is graphically displayed as a 'tree', with bifurcations depicting splits between populations).

Population variation

To investigate variation at the population level, P_{ST} values were calculated, and compared with F_{ST} values as found in the microsatellite analysis performed by van de Zande et al. (2000). Because we were not able to obtain F_{ST}-values directly in this study, we used estimates by van de Zande et al. (2000) instead, to give an indication. Those were obtained from populations from roughly the same regions as the samples in this study. The F_{ST} for their comparison between countries can only give an indication of the range in which the actual value for a comparison between Dutch and Finnish populations would be, since in their article, the comparison also involved populations from Norway and Germany. The calculation was done for pairwise combinations of populations, which were then ordered to the level of comparison, to calculate average P_{ST}.

Results

Geometric morphometrics

For the comparison between Dutch and Finnish populations, the Procrustes transformed landmarks for all individuals reveals clear shape differences. The Shape PCA revealed that the first two components explained 52.9% of all variation. These two principle components were then selected to perform subsequent analyses. A shape deformation plot from mean skull shape also suggests a difference in shape between the Finnish and the Dutch populations (figure 3), which is confirmed by a Hotelling's T^2 test indicating significant differences in scores on principle components 1 and 2 between Dutch and Finnish populations (P<0.0001).

Subsequently, regions within countries were compared, to identify possible differences on a regional scale. A plot of Procrustes transformed landmarks does not reveal any clear pattern, as there is considerable overlap between the shapes of skulls from different regions (plot not shown).

Nevertheless, MANOVA analysis of PC1 and PC2 indicated significant differences between regions ($F_{10,716} = 3.919$, $P < 0.0001$). To pinpoint the location of the differences in spe-

Table 1. Significant differences between Dutch regional clusters from Hotelling's T^2 test and sequential Bonferroni adjusted critical P-values (α). BB= Biesbosch, ZL = Zeeland, ZH = Zuid-Holland, TX = Texel.

Populations		P-value	Adjusted α
BB	ZL	0.000005	0.003414
BB	ZH	0.000109	0.003657
BB	TX	0.002972	0.003938

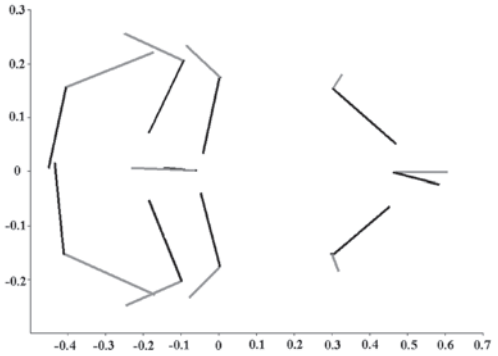

Figure 3. Plot of principal deformation from mean skull shape. Data for all skulls used in analysis. Lines indicate size and direction of the deviation for principal components 1 (grey) and 2 (black).

cific populations, a post-hoc Hotelling's T2 test was performed. This showed significant differences between regions BB-ZL, BB-ZH and BB-TX (table 1) after sequential Bonferroni correction for multiple testing. Thus, it appears that the Biesbosch region is significantly different in shape from the other Dutch regions. The Finnish populations were analysed in the same way as the Dutch for regional differentiation, but a MANOVA on the first two principle components indicated no significant differences between Finnish regions ($F_{6,70}$= 1.135, P = 0.3514).

The dendogram based on Mahalanobis distances reflects the significant differentiation between Finland and the Netherlands, and the significant differentiation of the Biesbosch region within the Netherlands. Differentiation of populations within Finland is also considerable, but not significant, most likely due to low sample size.

P_{ST} Values

Values of P_{ST} for comparisons between pairs of populations show that the P_{ST} between the Netherlands and Finland is larger (0.0471) than that between Finnish populations and that between Dutch populations separately (0.0224 and 0.0152 respectively). This suggests that the Finnish and Dutch populations are morphologically further apart than the populations in both countries are from each other. Furthermore, the average P_{ST} for the Finnish populations are higher, which would concur with the fact that the populations are separated by greater distances, and perhaps have been separated for longer periods of time, than those in the Netherlands.

Comparison of genetic and morphological divergence

F_{ST} values reported by van de Zande et al. (2000) are higher than the P_{ST} values found in this study. For differences between regions in the Netherlands they found an average F_{ST} of 0.1582 (95% confidence interval 0.1323-0.1840). Between countries, the average F_{ST} they found was 0.1708 (95% C. I.: 0.1415 to 0.2001). The difference between P_{ST} and F_{ST} ranges from three- (Dutch regions) to ten-fold (between countries). This suggests that the populations are under (strong) stabilising selection for skull shape.

Discussion

P_{ST}- F_{ST}

The calculated values for P_{ST} can be slightly inflated because phenotypic plasticity can have an influence on the variation measured: populations from different regions will experience different environmental conditions. This may affect phenotypic variance so that the between-population component increases. In other words, differences found between populations will not only represent the underlying genetic variation, but also environmental variation. This would increase variation between populations (V_B), which would thus increase the value of P_{ST}. On the other hand, our estimate of the within-population phenotypic variability V_w includes environmental variation and measurement error. The total

difference between the true value of P_{ST} and our estimate is determined by the, unknown, strength of these biases. Ideally, a multi-generation common garden experiment should be set up with animals from the different habitats in similar conditions to study the magnitude of the environmental influence on the phenotype, but such is difficult to achieve in practice. Despite these uncertainties, the difference between the P_{ST} values found and the F_{ST} values is three to ten-fold, making it unlikely that the conclusion that there is stabilising selection acting on the phenotype, would be altered. Apparently there is selection on an optimal phenotype for the separate habitats, making phenotype variation smaller than the neutral genetic variation.

Sampling bias

We do not know if and how the barn owls and long-eared owls selectively choose their prey in a way that is related to skull shape. This means that we are not entirely sure whether or not the sampling of skull shape was random: in theory, differences in skull shape between geographic regions that we reported could be due to differences in prey choice between owls from different regions. However, as Finnish populations (sampled by trapping) showed differentiation in skull shape to be comparable to variation in Dutch populations (sampled by owls), we believe that skull samples from owl pellets reliably describe differences between vole, not owl, populations. Second, we cannot be strictly sure whether one owl only sampled from only one population. However, for all analyses we grouped local populations in regions far exceeding the home range size of an owl, so that sampling from more than one population is not likely to be an issue.

Another problem could be the unknown age distribution of the root vole populations sampled. Since adult animals are larger than juveniles this could influence the analysis. However, geometric morphometrics studies mainly shape, and not size. And even though the shape of a skull or other skeletal features will change during ontogenesis, it is still possible to assess shape differences, even between adult and juvenile specimens (Marcus et al. 2000).

Similarly, it was not possible to discriminate between males and females based on the skulls alone, so possible sexual dimorphism could interfere with test results. Several other studies on morphometric analysis in rodents (Reutter et al. 1999, Barčiová & Macholán 2006) and also one other on *M. oeconomus* (Ràcz et al. 2005) found no sexual dimorphism, suggesting sexual dimorphism is low relative to total phenotypic variance. However, looking at specific age classes Markowski (1980) and Markowski & Østbye (1992) claimed evidence of sexual dimorphism in certain phenotypic characters of root voles, though without correcting statistically for testing a large number of characters. Thus, it was not possible to account for potential effects of age and sex on skull shape, but if such effects exist they are unlikely to have much effect on population comparisons using geometric morphometrics of skull shape.

Phylogeography

In the glacial periods up to the last glacial maximum (21,000-17,000 years ago), the root vole had a large habitat range in Europe, expanding its range further south than the current distribution. It is believed that there were several glacial refugia in central Europe (Chaline 1987). As the climate warmed, the population withdrew, leaving some populations isolated. The now isolated populations in the Netherlands, Slovakia and Hungary are very probably remnants of this larger range: analyses of mitochondrial DNA confirm this historical model, as these populations are part of the same mtDNA group (Brunhoff et al. 2003).

As temperatures rose after the ice-age, Scandinavia was released from its ice cover,

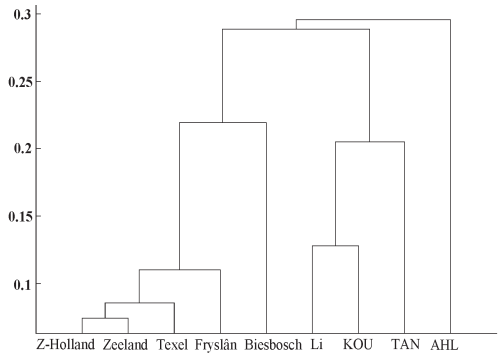

Figure 4. Dendrogram based on Mahalanobis distances between populations in the Netherlands and Finland. LI= Li, KOU = Kuusamo/Oulanka, TAN= Tankari, AHL = Ahlainen.

which made it possible for the root vole to recolonise Scandinavia (Brunhoff et al. 2003). Similar patterns have been observed in other mammals (Jaarola et al. 1999), and in particular a similar pattern has been found for field voles (Jaarola & Searle 2002), which are ecologically very similar to root voles. For the Finnish populations this means that the populations in the south may have become isolated from those in the north when the main population of root voles withdrew with the receding ice. If that scenario is correct, it is precisely reflected by the phenotypic distance tree (figure 4) which also shows an increasing phenotypic distance between populations with increasing latitudinal separation. The population in Ahlainen in southern Finland would have become isolated first, followed by Tankari in mid-Finland, and so on (see figure 1b).

The P_{ST} values, which are comparable with the Mahalanobis distance-based tree, support this. Also here, the further apart geographically the Finnish populations are, the larger the pairwise P_{ST} values are. For the Dutch populations, where the Biesbosch population differs significantly from Zeeland, Zuid-Holland and Texel, also the distance tree shows a split between the Biesbosch population and the others. This would mean that the Biesbosch population has been isolated from the others for a longer period of time.

Conclusions

Geometric morphometrics has proven to be a very powerful tool since it was possible to detect even small differences between populations, based on a limited number of landmarks from incomplete skulls. A dendrogram of population morphological differences (figure 4) is consistent with molecular phylogenies based on allozymes, and microsatellites (Leijs et al. 1999, van de Zande et al. 2000). On the small geographical scale of the Netherlands, morphological differences between populations exist. What was slightly unexpected is that the divergence between Dutch and Finnish populations based on morphological characters was smaller than the average F_{ST} from between-country comparisons by van de Zande et al. (2000). This suggests stabilising selection on skull shape for all populations, which keeps morphological variation low. Overall, our findings suggest that geometric morphometric analyses of skulls fragments obtained from owl pellets may provide a cost-effective, non-invasive tool to monitor subdivision of small mammal populations in fragmented habitats.

Acknowledgements: Many thanks to J. de Jong for providing samples and contacting volunteers. Thanks to W. Spijkstra, K. Mostert and J.P. Bekker for providing samples from the Netherlands and to Risto Tornberg from the University Natural History Museum in Oulu, for allowing the use of museum specimens. We would also like to thank an anonymous reviewer for useful comments on an earlier version of the manuscript. VvdB was supported by an Erasmus travel grant from the European Union for this study.

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Samenvatting

Verschilt de schedelvorm tussen geïsoleerde populaties van de noordse woelmuis (*Microtus oeconomus*) in Nederland?

We hebben verschillen in morfologie tussen verschillende populaties van de Nederlandse ondersoort van de noordse woelmuis (*Microtus oeconomus arenicola*) onderzocht. We hebben hierbij gebruik gemaakt van geometrische morfometrie-metingen aan woelmuizenschedels afkomstig uit braakballen van uilen. Daarnaast hebben we de gevonden morfologische differentiatie vergeleken met waardes van genetische differentiatie voor dezelfde populaties afkomstig uit de literatuur. Hierbij zijn de populaties uit Finland als referentie gebruikt. We vonden dat de morfometrische populatiedifferentiatie in het algemeen lager was dan de genetische, maar dat deze wel dezelfde patronen van geografische isolatie vertoonde. Dit suggereert dat de vorm van de schedel geconserveerd is in geïsoleerde woelmuizenpopulaties en dat geometrische morfometrische metingen van onderdelen van het skelet afkomstig uit uilenbraakballen een goedkoop alternatief kunnen zijn om subpopulaties van dezelfde soort te vergelijken.

Received: 26 April 2011
Accepted: 13 October 2011