Population genetic structure and genetic diversity of *Pipistrellus nathusii* along the Dutch coastline during the autumn migration period

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The current memo has a purpose to provide a (policy) summary of a paper that is currently in preparation (van Schaik et al, in prep).

Introduction

The development of offshore wind farms (OWF's) plays an important role in the Dutch energy transition to comply with the European aim to be climate neutral by 2050. There are however also concerns about the impact of (offshore) wind farms on biodiversity and protected species. The bat species Nathusius' pipistrelle (*Pipistrellus nathusii*) is one of these species of concern. This protected species is known to migrate from the breeding areas in eastern Europe towards their wintering areas in southern and western Europe. Some of them go as far as the UK and thus have to cross the North Sea during their migration (Russ, 2022, Lagerveld et al 2023). It cannot be ruled out that offshore wind developments at the southern North Sea may have a significant negative impact on this species (Leopold et al 2014). Within the Dutch Offshore Wind Ecological Programme (Wozep, executed by RWS) Nathusius' pipistrelle was therefore identified as priority species in studying the impact of the Dutch offshore wind development on bats.

The bat research programme within Wozep[1] includes various (sub)projects, all of which are aimed to fill important knowledge gaps and answer the overall research question: what is the relevance of the (presumed) mortality due to offshore wind farms for bats, in particular for Nathusius' pipistrelle population(s) crossing the southern North Sea. In order to perform an overall effect assessment, information is needed on:

1. The population size of the relevant source population(s)
2. The occurrence in space and time
3. The fatality risk of offshore wind turbines to bats

Limpens et al (2017) estimated that the relevant source population of Nathusius' Pipistrelle consists of approximately 275,000 individuals (bandwidth 25,000 – 2,000,000) of which approximately 40,000 individuals (bandwidth 100 – 1,000,000) may migrate over the southern North Sea. As the estimate was based purely on expert judgement in combination with the rather extensive confidence intervals, RWS felt the need to further investigate the population size. In addition, information on the population structure of Nathusius' pipistrelles residing and migrating through the Netherlands is lacking. Therefore, it is unknown whether individuals belong to one population, or that different (sub)populations are involved, each of which potentially needs to be treated as a separate entity for conservation purposes.

Population genetics can help characterize populations and movement dynamics. Modern techniques for analysing the genetic variation can help to get more insight into the population structure. It is possible to identify whether individuals belong to a large panmictic population, or whether the population is sub

[1] Bats - Noordzeeloket UK

structured. Also, monitoring of genetic diversity metrics such as heterozygosity, allelic richness, and effective population size estimates can help infer population trends. Finally, it can also be used to learn more about the social structure and life history of species, e.g. the genetic relatedness of individuals sampled together.

Aim of the study

RWS has asked Wageningen Marine Research to execute a research project where genetic samples of Nathusius' pipistrelle found in the Netherlands are taken and analyzed aiming to answer the following research questions:

1. Is the (meta)population that migrates through the Dutch coastal provinces structured; can subpopulations be identified?
2. What is the 'effective population size' of the relevant (sub)population(s)?
3. Do females guide their offspring during migration?

Approach

During four consecutive years (2020-2023) between August and October WMR collected 448 genetic samples in the Dutch coastal provinces South Holland, North Holland and Friesland (Supplementary information: S5 Pnat Genotype Full - van Schaik et al, in prep), which were subsequently analysed and interpretated by the University of Greifswald to answer the research questions above. The results are summarized in a manuscript to be published in a scientific journal (target journal Conservation Genetics).

Results

It was found that the population is panmictic (not structured), with an effective population size estimate in the hundreds of thousands that cannot be distinguished from a population of infinite size (estimate: 198.229, confidence interval: 7721-infinite).

The effective population size represents the size of an idealised population that would experience the amount of genetic drift observed in the sampled population. In an infinitely sized population, any chance events, such as the unexpected mortality of some individuals prior to reproduction, will not change the allele frequency and expected heterozygosity of the population (i.e. all alleles will be inherited at the same frequency as they occur in the previous generation; known as Hardy-Weinberg equilibrium). In most large populations (>10,000), the overall genetic drift in allele frequencies is generally lower than the noise induced by sampling only a small fraction of the population, and thus the confidence interval of effective population size estimates from such populations nearly always includes infinity (e.g. Waples 2024). As such, the observed result where the confidence interval of the effective population size includes infinity is perhaps unsurprising, although the results would obviously have been different had the population been sub-structured.

Comparing the genetic diversity of samples of four consecutive years, two factors suggest an ongoing population decline; first, a subtle but consistent decline in allelic richness was observed (2.5% between the first and last year). Although not statistically significant, it nevertheless potentially represents a considerable decline in overall population size. Second, a marked trend towards heterozygote excess in both juvenile males and females was observed. When a population experiences a substantial reduction in size, allelic diversity is reduced faster than heterozygosity. As a result, the observed heterozygosity will temporarily be higher than would be expected given the observed number of alleles. If the population stabilizes, this excess will gradually disappear as the population returns to mutation-drift equilibrium (Cornuet & Luikart 1996). Thus, the heterozygote excess observed here, while again statistically insignificant, may point towards a recent population decline.

We did not find any parent-offspring pairs in our dataset, which included 30 box captures where adult females and juvenile bats were found roosting together, suggesting that juvenile bats do not follow their mothers during their first migration.

Relevance of the outcomes of this study for WOZEP

The results of this study indicate that Nathusius' pipistrelles residing or migrating through the Dutch coastal zone belong to a single panmictic population. Therefore, one population should be taken into account for management and in the overall effect assessment of offshore wind developments in the Netherlands. This implies that:

1. There is no need to consider various sub-populations as independent management units, which makes it easier to monitor the population trend.
2. Mortality in the Netherlands will affect the species throughout the entire catchment area of the investigated migratory pathway, and vice versa.

The current effective population size estimate puts the population at a similar order of magnitude as an earlier estimate of the population size based on expert judgement (Limpens et al. 2017). We have shown that it is plausible that the population is decreasing. This somewhat contradicts the most recent population trend estimate published by the CBS (Centraal Bureau voor de Statistiek), based on acoustic monitoring of standardized routes, which considers the population trend to be stable. However, it is widely acknowledged that inferring population trends from acoustic data is challenging (Marques et al. 2013), especially for migratory species, whose presence and activity may fluctuate strongly and may not be reliably characterized by incidental acoustic survey efforts.

Indeed, the published trend for *P. nathusii* fluctuates strongly from year to year (Figure 1), including several increases and declines that seem biologically implausible (i.e. a near doubling of the population from 2021 to 2022). Moreover, had the trend been analysed for 2015-2021 (ie. without 2022), the trend would have almost certainly been strongly declining.

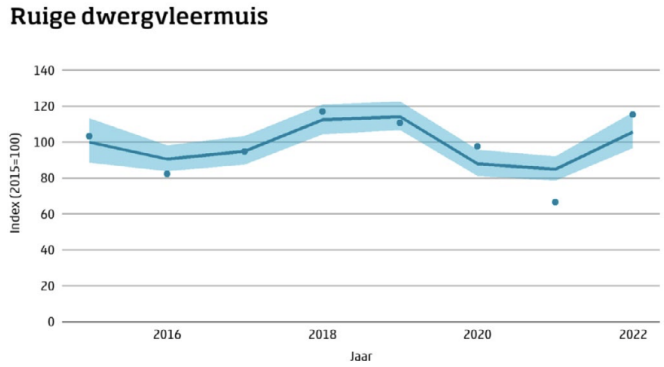

Figure 1: Population trend Nathusius' pipistrelle 2015 – 2022 in the Netherlands (Zoogdiervereniging, 2023)

The genetic diversity metrics investigated here also have their limitations, but may provide a more robust signal then the method used thus far. Genetic diversity is only replenished through mutation, which is a comparatively slow process (hundreds/thousands of years for any given locus), and through immigration of novel genotypes from other populations. As a result, monitoring genetic diversity will generally be unsuitable to detect population growth over short timescales (years/decades), as this will not immediately lead to an increased diversity (i.e. genetic diversity will remain stable). However, given an initial population with a high standing genetic diversity as observed in *P. nathusii*, population declines

will most certainly lead to the gradual loss of the existing diversity. As such, monitoring efforts that track the overall genetic diversity of the population over time can provide reliable indications of population decline when they occur.

As Nathusius' pipistrelle is one of the species with the highest observed number of fatalities at wind energy facilities (Dürr 2023), it is probable that wind energy developments across the species' range contribute to the (likely) decline. Therefore, the population is likely vulnerable to additional mortality, for example as a result of future wind energy developments, both on land and at sea.

The fact that we found no mother-offspring pairs in the dataset suggests that migration behavior is likely to be innate (ie. follow a genetically pre-determined migratory vector), as observed in many migratory birds (e.g. Berthold 2001). The lack of active mother-offspring guidance does suggest that juveniles are able to complete their migration, even if their mother dies before or during the migratory phase. On the other hand, even if bats adjust their movements to avoid wind farms over the course of lifetime based on personal experience, it appears that juveniles are unlikely to learn that from 'experienced' adults. Thus, it is expected that each new offspring cohort will be vulnerable to wind farms. Indeed, field research shows that more juveniles are killed than adults compared to their relative abundance in the population. (Kruszynski et al 2021).

Conservation Implications and Recommendations

As summarized above, the observed population structure and potential declining trajectory of the population, represent a scenario in which genetic population monitoring can be used to detect substantial declines in population size, when they occur. As monitoring the population trend of the species remains very difficult through other methodologies (ie. counts, acoustic monitoring), genetics therefore represents an appealing proxy that can be used to infer the degree to which additional mortality due to wind turbines and other anthropogenic causes are affecting the population. We therefore advocate for the establishment of a recurring sampling and analysis scheme that tracks genetic diversity of the population using the methods established in this study.

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Diverse but declining: Population genetic structure and genetic diversity of Nathusius' pipistrelle along the Dutch coastline during the autumn migration period

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Abstract

Migratory bats are experiencing substantial increases in mortality risk from wind energy developments, but data on their migratory behavior and population dynamics are often lacking. Here, we develop a novel microsatellite panel for one such migratory bat species, the Nathusius' pipistrelle (*Pipistrellus nathusii*), and apply it to 448 samples collected at stopover sites along the Dutch coast during autumn migration over four consecutive years. With this dataset, we assessed whether the population is genetically sub-structured, characterize its current genetic diversity, and evaluate whether mothers guide their offspring during migration. We found that the population is panmictic and diverse, with an effective population size estimate that cannot be distinguished from infinite. However, we also observed a consistent decline in allelic richness across the sampling period, as well as a heterozygote excess in individuals sampled as juveniles, both suggesting an ongoing population decline. We did not find any parent-offspring pairs in our dataset, which included 30 box captures where adult female and juvenile bats were found roosting together, suggesting that juvenile bats do not follow their mothers during their first migration. Our findings provide an initial characterization and baseline measure of genetic diversity for the Nathusius' pipistrelle that can be used as a reference for subsequent studies and systematic efforts to monitor the genetic diversity of the species. Given that monitoring population trends of migratory bat species with traditional methods remains challenging, such tracking of genetic diversity may offer a valuable proxy by which to observe substantial population declines if they occur.

Keywords: bat conservation, Chiroptera, *Pipistrellus nathusii*, wind energy, microsatellite, genetic monitoring

Introduction

In order to protect threatened species, it is pivotal to identify and preserve their habitats (Hoffmann et al. 2008). For migratory species, this encompasses both their summer and winter habitats, as well as the migratory routes between them (e.g. CMS 1979). For many migratory birds and bats, the rapid expansion of wind farms, especially along coastlines, at sea, and in narrow landscape corridors are making these migratory pathways increasingly more difficult to navigate. Indeed, it is estimated that hundreds of thousands of bats are killed annually by wind turbines (Hayes 2013; Voigt et al. 2015; Măntoiu et al. 2020), with the highest fatality rates occurring in migratory species during the migratory period (Kunz et al. 2007; Rydell et al. 2010). As such, there is an urgent need to assess the extent to which these developments threaten the viability of the affected populations (Frick et al. 2017), and to characterize the migratory behavior of these species in order to establish evidence-based action plans (Voigt 2020).

In Europe Nathusius' pipistrelle (*Pipistrellus nathusii*) is one of the species with the highest observed number of fatalities at wind turbines (Dürr 2023). Female maternity colonies of this small (6-10g) bat species are found throughout Central and Northeastern Europe (Russ 2022). Here, females give birth, often to two offspring (Vierhaus 2004). While some populations at the southern edge of the range are presumed to be sedentary, most populations migrate in a southwesterly direction in autumn, sometimes in excess of 2000km (Alcalde et al. 2021; Vasenkov et al. 2022). During summer, males establish and defend mating roosts along the migratory routes (Gerell-Lundberg & Gerell 1994). In autumn, females and juveniles of both sexes join males at these mating roosts to form temporary harems of up to a dozen individuals for one or more days (Gerell-Lundberg & Gerell 1994). In winter, individuals hibernate

86 solitarily or in small groups in minimally insulated roosts (e.g. crevices in buildings,
87 stacks of firewood; Gebhard 1997; Russ 2022).

88 Over the past decade, acoustic monitoring, analysis of wind farm fatalities, and
89 telemetry studies have improved our understanding of the migratory behavior and
90 routes used by Nathusius' pipistrelle. A diversity of migratory pathways has been
91 identified or suggested across Europe (e.g. Russ 2022), notably including several that
92 cross both the Baltic and North seas, where wind energy activities are expanding
93 rapidly. Rydell and colleagues (2014) found that the species migrates across a broad
94 front, with activity concentrating along coastlines and large rivers, but also over open
95 sea throughout the Baltic and southeastern North Sea. Likewise, an increasing number
96 of records from the United Kingdom (NNPP 2022) suggests that a proportion of the
97 population crosses the southern North Sea, as additionally supported by offshore
98 acoustic monitoring (Brabant et al. 2019, Lagerveld et al. 2021, 2023). An analysis of
99 wind turbine fatalities in Germany found that juveniles and female bats may be killed
100 at a higher rate, but did not find evidence that migratory individuals were at higher risk
101 than those from local populations (Kruszynski et al. 2022). Despite these advances,
102 the overall population size and population dynamics remain poorly characterized as
103 traditional census methods are inefficient due to the dispersed nature of the population
104 (Frick et al. 2017), and the ability to generate population trends from acoustic and
105 tracking data remains elusive. As a result, effect assessments that aim to understand
106 the potential risks posed by new wind farms cannot be adequately carried out.

107 The application of population genetic approaches can help characterize
108 population structure, trends and movement dynamics. At the most basic level,
109 population genetic analyses can establish whether the sampled individuals belong to
110 a large panmictic population, or whether the population is composed of genetically

111 differentiated sub-populations. Moreover, genetic diversity metrics such as
112 heterozygosity, allelic richness, and effective population size estimates can help infer
113 population trends (Schwarz et al. 2007; Willi et al. 2022). In this context, repeated
114 sampling of the population can be an especially powerful tool to reveal population
115 declines through losses in genetic diversity (Hoban et al. 2014) and increased genetic
116 drift. Such metrics can subsequently be incorporated into population viability analyses
117 to inform conservation measures (Frankham et al. 2014). Finally, kinship analyses can
118 help elucidate the social structure of a species, and can be used to assess the degree
119 to which individuals captured together are genetically related (e.g. Stumpf et al. 2017).
120 To date, the population genetic structure of Nathusius' pipistrelle has not been
121 investigated, and no marker panels for estimating and tracking temporal patterns of
122 genetic diversity exist.

123 Here, we present a novel panel of 21 microsatellite loci for Nathusius' pipistrelle,
124 and use it to investigate the population genetic structure and relatedness of 448
125 individuals sampled at nine coastal locations in the Netherlands between 2020 and
126 2023 during the autumn migration period. Specifically, we first investigated the genetic
127 sub-structuring of the collected samples using both Bayesian (Structure) and k-means
128 clustering approaches. We predicted that since individuals are mating in the sampled
129 stopover habitats, that the population will be unstructured, although cryptic population
130 structuring could still exist through assortative mating in sympatry. Subsequently, we
131 characterize the allelic richness, heterozygosity, F_{IS} and effective population size
132 estimates of the sampled population, and compare estimates across years and
133 between bat sex and age classes. Tracking such diversity metrices over time will help
134 evaluate the extent of the presumed ongoing population decline, although we did not
135 expect to observe a statistically significant decline within the four year period sampled

here. Finally, we investigated the pairwise relatedness of all samples and performed parentage analysis to search for parent-offspring pairs across the entire dataset. We hypothesized that since adult females and juveniles (sensu young-of-the-year) were often recorded to roost together at the sampled stopover sites, juveniles might follow their mothers during their first migration, as has been suggested at a more local scale (<50km) between summer habitats and hibernacula in Natterer's bats (Stumpf et al. 2017). In this case, we would expect to recover mother-offspring pairs originating from samples taken concurrently, especially amongst adult-juvenile pairs sampled simultaneously from the same bat box. Moreover, if the two offspring raised by a mother during a summer migrated together, we would expect that pairs of juveniles sampled together would be related at the half- or full-sib level.

Material and Methods

Bat Capture and DNA Extraction

A total of 448 *Pipistrellus nathusii* samples were collected (Table 1). Samples were collected in nine forest patches distributed across three Dutch coastal provinces (Friesland, North Holland and South Holland) between August and October over the course of four years (2020-2023). Two different capture methods were used: box captures and mist-netting. For the first, artificial bat boxes (Model types: Schwegler 2FN and 1FF, Vivara PL 01, and 'Boshamer' type 1 and 2) were checked for the presence of *P. nathusii* during the day. If present, the bats were removed from the box, processed, and returned to the box within 60 min of capture. This yielded 211 samples, including 110 samples from 30 boxes where adult female and juvenile bats were caught together from the same box. For the second method, mistnets (Ecotone, Gdynia, Poland and Solida Safety Line, Helmstedt, Germany) and three-bank harptraps (Faunatec Austbat, Victoria, Australia) were placed at night. Here, bats were removed from the net or harptrap, processed, and released within 30 min of capture. For all bats, processing involved measurement of forearm length, weight, and determining the sex and age. Age was scored as either adult or juvenile (sensu Young-of-the-year; scoring as in van Schaik et al. 2015). Full capture information for each individual can be found in the Table S5. All capture and sampling were performed under license (Capture and handling: permit no. 2018-057682; DNA sampling: AVD248002016459 / VZZ-18-005 and AVD24800202114476 / VZZ-2021-001).

A 3-mm wing-punch was taken for genotyping (Wilmer & Barratt 1996). Wing-punch samples were preserved in 70% ethanol prior to DNA extraction. Total genomic DNA was extracted following a salting-out extraction method using 4M ammonium

acetate precipitation method. Extractions were eluted into 70µl of Low TE-Buffer, and stored at -20°C prior to genotyping.

Table 1: Overview of the nine sampling sites, including the number of samples collected at each site across the four year study period. Of the 448 samples, two failed to amplify (from Wildrijk 2021, Kornwerderzand 2022), and the second sampling of eight recaptures (see Table 3) were also removed, yielding a final sample size of 438 individuals.

Province	Location	Lat/Long	2020	2021	2022	2023	Total
Friesland	Zurich	N 53.10 E 5.39		4			4
	Kornwerderzand	N 53.07 E 5.34		15	42	26	83
North Holland	Noorderhaven	N 52.88 E 4.76	12	24	20	14	70
	Callantsoog	N 52.84 E 4.71	13	19	25	2	59
	Wildrijk	N 52.79 E 4.70	4	43	42		89
	Ananasbos	N 52.80 E 4.73				34	34
	‘t Zand	N 52.85 E 4.77	6				6
South Holland	Hoek van Holland	N 51.99 E 4.12	30	6			36
	Goeree	N 51.66 E 4.26				67	67
		Total	65	111	129	143	448

Microsatellite development and genotyping

A microsatellite library was generated using next-generation sequencing (MiSeq Nano v2) based on a pooled sample containing equal DNA concentrations from eight samples (1 µg total weight; performed by Genoscreen, Lille, France). This resulted in 45,900 merged reads, which were screened for the presence of microsatellite repeat motifs, resulting in 169 potential primer pairs (QDD v3; Meglécz et al. 2010). Subsequently, 50 primer pairs were selected and ordered as unlabeled primers based

190 on expected product length, compatibility of the annealing temperatures of the forward
191 and reverse primer, and the number of observed repeats in the microsatellite motif.
192 The ordered primers were first evaluated for successful amplification on a
193 pooled sample of six individuals. The 5µl PCR reactions contained 1 µl of DNA, 2.5µl
194 Type-IT PCR-mix (Qiagen), and 1.5µl of both primers diluted to 0.2µM. PCR reactions
195 consisted of an initial denaturation at 95º C for 5min, 35 cycles of denaturation at 95º
196 C for 30s, primer annealing at 58º C for 90s, and elongation at 72º C for 60s, and a
197 final elongation of 60min at 60º C. Products were evaluated on a 1.5% Agarose-gel
198 using GelRed® (Biotium Inc.) staining. Those that amplified successfully were
199 subsequently run for four individual samples (2 male, 2 female) using the same
200 procedure to evaluate potential diversity, secondary amplifications and consistency of
201 amplification. Thirty primers were subsequently ordered with a fluorescent label for
202 further testing.
203 Fluorescent primers were evaluated using the same PCR conditions and same
204 four individuals. In this round, 1 µl of product was combined with 9µl of Formamide and
205 0.2µl of GeneScan LIZ500 size standard (Applied Biosystems) and visualized using an
206 ABI3130 (Applied Biosystems). In addition to the 30 primer pairs described above, 60
207 primer pairs for microsatellite loci developed for other bat species that were available
208 as fluorescently labeled primers in our lab were similarly evaluated (*Myotis bechsteinii*:
209 van Schaik et al. 2018; *M. nattereri* and *M. daubentonii*: Stumpf et al. 2017; Other:
210 Castella & Ruedi 2000, Miller-Butterworth et al. 2002, O'Donnell et al. 2016). In total,
211 25 microsatellite loci (21 newly developed loci, 4 previously established for other
212 species) were selected for analysis and divided into four multiplexes (Table 2). PCR
213 conditions and fragment analysis for multiplex amplification were identical to the

conditions described above for primer testing. Scoring of microsatellite loci was performed in Genemapper (v5; Applied Biosystems).

Statistical analysis

All statistical analyses were performed in R (v4.3.2; R Core Team 2023) unless otherwise noted.

After initial scoring, raw fragment sizes and allele calls were plotted to check for scoring mistakes and allele call consistency. Markers were evaluated for deviation from Hardy-Weinberg equilibrium (implemented in Pegas v1.3; Paradis 2010), and pairwise index of association ($\bar{r}_d$ using the pair.ia function in poppr v2.9.6; Kamvar et al. 2014) was calculated to evaluate linkage disequilibrium (Agapow & Burt 2001). For both tests, the P-value was adjusted for multiple testing using the Benjamini-Hochberg method (Benjamini & Hochberg 1995). The frequency of null alleles was assessed using the Brookfield method (Brookfield 1996), implemented in PopGenReport (v3.1; Adamack & Gruber 2014). To confirm that the microsatellite panel was appropriate to distinguish individual genotypes, we calculated the probability of identity between siblings (P_{IDsibs}; Waits et al. 2001). To check for recaptures, we searched for duplicate genotypes using the mlg.id function in poppr (Kamvar et al. 2014). For recovered duplicates, the second sampling of an individual was removed from the dataset prior to further analysis.

Genetic population structure was inferred using two approaches. First, population sub-structuring was evaluated using a Bayesian clustering approach, implemented in Structure (v2.3.4; Pritchard et al. 2000). We evaluated a range of potential subpopulations (K) from 1 to 4, with 5 iterations per K, where each run consisted of a 200,000 step burn-in and a run length of 500,000 steps. We used an

admixture model, with uncorrelated allele frequencies between populations, without using location information as a prior, and an individual alpha for each population (initial alpha: 0.1). Support for the most likely number of clusters was estimated using the log-likelihood method, implemented in StructureSelector (Li & Liu 2018). Second, we performed K-means clustering (Jombart et al. 2010), as implemented in adegenet (v2.1.10; Jombart 2008). Here, BIC values were inspected for between 1 and 20 clusters with 100 PCs retained for initial inference. We subsequently used a discriminant analysis of principal components (DAPC) to visualize the inferred clusters at the best K value, using the optim.a.score function to determine the number of retained principal components for the visualization.

Genetic diversity metrics were generated for 1) the full dataset, 2) per sampling year, and 3) between sex and age classes (ie. adult male, adult female, juvenile male, juvenile female). For each dataset, the number of alleles per locus, the allelic richness, expected and observed heterozygosity, and F_{is} values were calculated using the hierfstat package (v0.5-11; Goudet et al. 2005). For the second and third datasets, the number of private alleles per class was calculated in PopGenReport (Adamack & Gruber 2014). Effective population size was estimated using NeEstimator v2 (Do et al. 2014) using the Linkage Disequilibrium method with a minimum allele frequency threshold of 0.01 and a 95% confidence interval calculated using the parametric method.

Pairwise relatedness was calculated in related (Pew et al. 2015) using the Wang relatedness estimator (Wang 2002). In the initial analysis, we calculated the pairwise relatedness of every pair of individuals in the dataset. Next, to test whether adult females and juveniles residing in the same box were more related than average, we then subset all adult female-offspring pairs caught together and compared their

relatedness to that observed across the whole population. Finally, to evaluate whether juveniles potentially migrate together with their half- or full-sibling, we similarly subset the cases where two or more juveniles were found residing in the same box.

Parentage analysis was performed in Cervus (v3.0.7; Marshall et al. 1998). All adult males and females were considered as potential parents (82 males, 171 females) and all juveniles (n=193) as potential offspring. We simulated 100,000 offspring, with a 1% genotyping error rate, and 99.5% of loci typed. The estimated proportion of candidate mothers and fathers sampled in the dataset was intentionally overestimated (15%) to ensure that even potential pairs with several mismatches would be reported. All pairs with a positive LOD score were evaluated and considered true parent-offspring pairs if they had 0 or 1 mismatches across all loci.

Table 2: Overview of the 21 microsatellite loci analyzed in this study. For each locus the following information is provided: locus name, the primer sequences (Forward / Reverse), the source and GenBank Accession number of the amplified fragment, the fluorescent label used (Fl. label), the size range of the amplified fragment, which multiplex the locus was run in (Multiplex) along with the final primer concentration in the PCR (PCR Conc.), the repeat motif being amplified, the number of observed alleles (No. Alleles), the observed and expected heterozygosity (H_e and H_o), and the F_{is} value per locus.

Locus	Forward / Reverse (5' - 3')	Source / Accession No.	Fl. label / Size range	Multiplex / PCR Conc.	Repeat Motif	No. Alleles	H_o	H_e	F_{IS}
PiNa09	F: TGACATCATTACCCTGCCGG	this study	FAM	3	$(AGAT)_7$	20	0.849	0.859	0.013
	R: TCTCAGGATGCAGTTTGTGGA	PQ641316	110-170	0.15µM					
PiNa12	F: ACCCACTAATCTATCTTACCCATCC	this study	VIC	1	$(AGAT)_{13}$	10	0.813	0.768	-0.058
	R: TCCCACTGCTGAAAGATGGA	PQ641317	90-120	0.1µM					
PiNa15	F: ACCTCTAGTGCCTGAAAGACA	this study	FAM	1	$(AC)_3AT(AC)_{16}$	15	0.716	0.730	0.020
	R: GTGAGAAGCCAAGTCCCACA	PQ641318	140-180	0.1µM					
PiNa16	F: GGACAAGCCTTCAGCCAACT	this study	PET	2	$(AAT)_{10}$	7	0.767	0.799	0.041
	R: TTAGATGCAACCCAGGTGCC	PQ641319	160-210	0.22µM					
PiNa19	F: CCCATATGACCCAATGGCCA	this study	FAM	2	$(AC)_{10}(CA)_6CG(CA)_5$	13	0.815	0.802	-0.015
	R: TGCCTCTGTTAGCCATATCTCAG	PQ641320	160-200	0.2µM					
PiNa20	F: TCACAGATCTGATGAGCCAGT	this study	PET	4	$(AT)_{23}$	22	0.874	0.886	0.015
	R: CAGGGTTTCCAACATGTGACA	PQ641321	140-210	0.45µM					
PiNa21	F: CCTCCTCTAGTCTTTGGAAGGG	this study	NED	1	$(AC)_{20}$	22	0.879	0.866	-0.014
	R: GCTGCAATCCCAGAACTCCT	PQ641322	160-210	0.2µM					
PiNa24	F: TACGTTGCTGTTTAGAATGACTAGT	this study	VIC	2	$(AC)_{11}$	7	0.605	0.604	-0.001

	R: TTCATAAGGAAAGCAGGGCACT	PQ641323	180-200	0.22μM					
PiNa25	F: GCCTCAAATATCACTAGTGCTGC	this study	FAM	3	$(ACT)_{13}$	13	0.685	0.689	0.008
	R: CACATATGCGGGTCCCAGAT	PQ641324	170-210	0.25μM					
PiNa27	F: ACAGGGAACTCATAGTCTTGGC	this study	FAM	4	$(AAAT)_{10}$	15	0.831	0.806	-0.030
	R: GTTCCATGCCTGTGTCTGCT	PQ641325	180-220	0.05μM					
PiNa32	F: GGTGCTGTGAATGAGAAGGC	this study	FAM	1	$(AC)_{18}$	15	0.861	0.882	0.025
	R: GACAGGTTGCAGTAGCTGGT	PQ641326	200-250	0.2μM					
PiNa33	F: ACCCTTCAGAGCATAGTTAAGGC	this study	PET	2	$(AC)_4CC(AC)_{11}$	15	0.856	0.883	0.032
	R: GAAAGCGACAGGAGAGGAGC	PQ641327	230-270	0.5μM					
PiNa35	F: GCACCTTTGAGCAACTGGTG	this study	VIC	3	$(AC)_{21}$	40	0.918	0.924	0.008
	R: CACTCCCTGAATTCCAGCAGA	PQ641328	200-240	0.3μM					
PiNa36	F: GTCTGGGCCTTTGGACTGAA	this study	PET	3	$(AGAT)_7$	18	0.801	0.819	0.022
	R: CCTCAGGGTTAGAGTGCTGT	PQ641329	230-290	0.25μM					
PiNa38	F: ACCCAAGTAAGGAGCATGCA	this study	VIC	2	$(AT)_7$	5	0.548	0.524	-0.044
	R: CAAAGTCGTCTTATATGCCGGA	PQ641330	240-260	0.22μM					
PiNa45	F: CCACCGGCTGATCTAATTAGCA	this study	VIC	1	$(AC)_{20}$	15	0.845	0.844	0.000
	R: TCAGGTTTACCAGAGCACGG	PQ641331	240-290	0.2μM					
PiNa48	F: ATGTGACTAGGGCTGCTTGG	this study	FAM	2	$(AC)_{17}$	17	0.884	0.879	-0.004
	R: ATCACAACCACTGGAGCATCA	PQ641332	280-320	0.45μM					
G6	F: GGCTTTTTGAAAAGACTGAGG	Castella & Ruedi 2000	PET	3	$(GT)_{12}$	21	0.900	0.897	-0.002
	R: ACATCAGCCAGTTCCTGTTC	AF203665	90-140	0.1μM					
GTUN9	F: AATGAAGCAAAGAGAAACAATGG	O'Donnell et al. 2016	VIC	3	$(AC)_{12}$	17	0.918	0.912	-0.005
	R: GTTTC-TGGAAACTTGGAAATGTGACC	KT013260	120-170	0.05μM					
GTVIA	F: ACAGCTGCCAGGAATCTGAC	van Schaik et al. 2018	NED	4	$(CA)_7CG(CA)_{10}$	15	0.852	0.858	0.009
	R: TGACCCAGTCTCCTCCAAAG	MG321325	170-210	0.1μM					
Mschreib3	F: AGCCAGGCACAGCTCAC	Miller-Butterworth et al. 2002	NED	4	$(CA)_{19}$	34	0.920	0.917	-0.002
	R: GTTTTC-TTTGGCATCTGAAGG	AY056590	240-300	0.25μM					

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Results

Marker characteristics

In total, 446 of the 448 samples were successfully amplified, with a maximum of one locus missing per individual (overall missing data: 0.05%). Four loci of the 25 loci that were included in the multiplexes could not be consistently scored or showed significant homozygote excess (Table S1), and were thus excluded from the analysis. None of the remaining 21 loci deviated significantly from Hardy-Weinberg equilibrium (Table S2), showed signs of null alleles (Figure S1), or were significantly linked (max $\bar{r}_d$ = 0.035; Table S3). Summary statistics per locus are provided in Table 2.

Recaptures

We recovered 8 duplicate genotypes (Table 3). Given the low probability of identity across all loci (P_{IDsibs} = 3.09×10^{-10}), these were considered recaptures of the same individual. All recaptures occurred within the same sampling location, with 7 of 8 recaptures occurring in different years. Five of the 8 recaptured individuals were male, of which two were captured from the same bat box in consecutive years (individuals 4 and 5; Table 3). One adult female was recaptured within the same year, 49 days after initial capture (individuals 6, Table 3). No note was made of an existing hole or scar in the wing tissue, suggesting the wing-punch had fully healed by the time of the second sampling event.

Table 3 Overview of all individuals that were recaptured, as determined through perfect genetic match of the samples, during the study. Location names correspond to those given in Table 1.

Pair	Location	Capture method	Date	Age	Sex
1	Noorderhaven	Box	25.08.2020	Ad	Male
1	Noorderhaven	Box	23.09.2021	Ad	Male
2	Hoek van Holland	Box	25.08.2020	Ad	Male
2	Hoek van Holland	Net	22.09.2021	Ad	Male
3	Callantsoog	Box	13.09.2020	Ad	Male
3	Callantsoog	Box	17.09.2021	Ad	Male
4	Wildrijk	Box*	10.10.2021	Juv	Male
4	Wildrijk	Box*	25.09.2022	Ad	Male
5	Wildrijk	Box*	09.09.2021	Ad	Male
5	Wildrijk	Box*	31.08.2022	Ad	Male
6	Hoek van Holland	Box	25.08.2020	Ad	Female
6	Hoek van Holland	Net	13.10.2020	Ad	Female
7	Hoek van Holland	Box	15.09.2020	Ad	Female
7	Hoek van Holland	Box	15.10.2021	Ad	Female
8	Noorderhaven	Box	23.09.2021	Juv	Female
8	Noorderhaven	Net	02.09.2023	Ad	Female

* denotes that the individual was captured from the same bat box both times

Genetic diversity

The Structure analysis showed highest log-likelihood support for a single population (K=1), with higher values of K only partitioning small fractions of individual ancestry into additional clusters (Figure S2). The k-means clustering analysis found highest support (lowest BIC value) for 3 clusters, however when visualized in a DAPC, the three clusters were not spatially segregated and formed a single cluster divided into equal thirds (Figure S2). Taken together, we therefore conclude that all samples likely belong to a single panmictic population.

Across the full dataset, we recovered a diverse (average alleles per locus = 16.95), and well-mixed (FIS = 0.001) population, that could not be distinguished from an infinitely large population (N_e = 198,229, range = 7721-∞; Table 4). When we subdivided the samples by sampling year, metrics were broadly similar over time, with a subtle consecutive decline in allelic richness over the four year sampling period (from 12.816 to 12.489; Table 4). When considered per bat sex and age class, we observed a notable heterozygote excess in both juvenile classes and homozygote excess in both adult classes, although all four were statistically insignificant as indicated by the inclusion of 0 in the 95% confidence intervals (Table 4).

328 **Table 4** Genetic diversity metrics for *Pipistrellus nathusii* captured along the Dutch
329 coastal provinces during autumn migration between 2020-2023. Metrics are provided
330 for the whole population (top line) as well as per year and per bat sex and age class.
331 Abbreviations: N, sample size; Na, average number of alleles/locus; K, allelic richness;
332 Priv All, number of private alleles, H_o, observed heterozygosity; H_e, expecteded
333 heterozygosity; F_{is}±CI, inbreeding coefficient with 95% confidence interval; N_e±CI,
334 effective population size with 95% confidence interval
335

Population	N	Na	K	Priv All	H_o	H_e	F_{is}±CI	N_e±CI
Full population	438	16.95	16.944	NA	0.816	0.818	0.001 (-0.009 - 0.011)	198229 (7721-∞)
Per Year								
2020	64	12.86	12.816	11	0.829	0.819	-0.013 (-0.033 - 0.007)	10350 (802-∞)
2021	106	13.86	12.605	17	0.813	0.819	0.007 (-0.009 - 0.021)	22881 (1898-∞)
2022	126	14.05	12.595	16	0.811	0.817	0.005 (-0.015 - 0.026)	∞ (2982-∞)
2023	142	14.24	12.489	12	0.816	0.818	0.001 (-0.012 - 0.018)	∞ (16385-∞)
Per Class								
Adult male	77	12.81	12.784	7	0.809	0.823	0.015 (-0.007 - 0.039)	∞ (3209-∞)
Adult female	168	14.81	13.035	20	0.807	0.816	0.012 (-0.005 - 0.027)	∞ (18863-∞)
Juvenile male	93	13.71	13.245	9	0.829	0.818	-0.015 (-0.033 - 0.007)	∞ (3829-∞)
Juvenile female	100	13.52	12.903	12	0.824	0.814	-0.014 (-0.032 - 0.008)	∞ (3800-∞)

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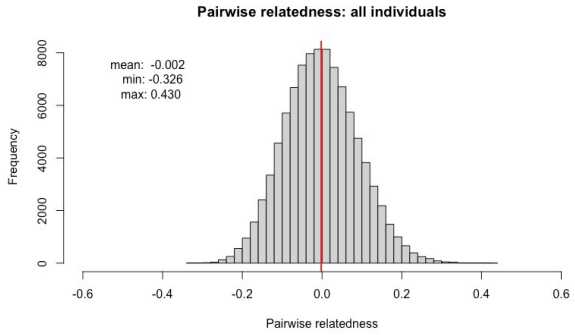
Pairwise relatedness and parentage

Average pairwise relatedness across the whole dataset was -0.002 (Figure 1a). The maximum observed pairwise relatedness across all pairs of individuals was 0.43, suggesting no direct parent-offspring or full-sib pairs. This was confirmed by the parentage analysis, which did not recover a single parent-offspring pair with less than 2 mismatches across all loci (see Table S4 for all pairs with positive LOD-score).

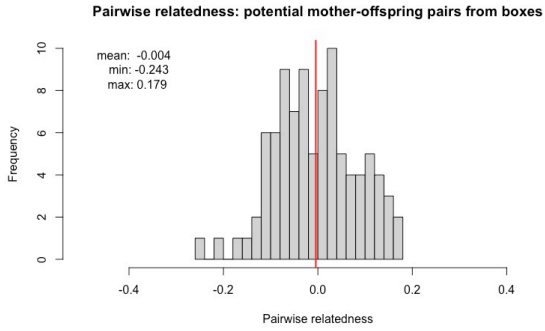
Similarly, no closely related pairs were observed when only considering the pairwise relatedness of adult females and juvenile bats that were captured together from the same box (n = 93; mean = -0.004; max = 0.179; Figure 1b). When comparing juvenile-pairs recovered from the same box, most pairs appeared similarly unrelated (mean = -0.0103; Figure 1c), however one pair was related at the half-sib level (relatedness: 0.2635; both juvenile females).

Fig. 1 Histograms of observed pairwise relatedness for a) the entire dataset (n=95,703), b) across all potential mother-offspring pairs that were sampled from within the same bat box (n=93), and c) across pairs of juveniles sampled from within the same bat box (n=43)

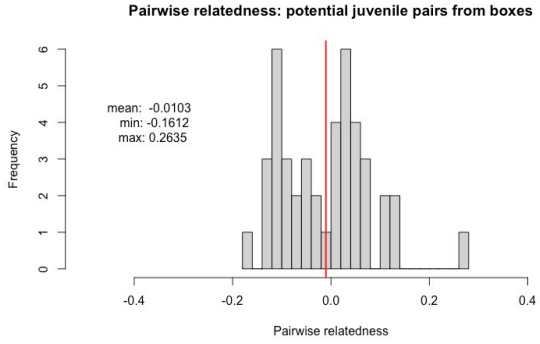
a)



b)



c)



Discussion

Characterizing the migratory behavior, population dynamics and current genetic diversity of migratory species is urgently needed in the face of the existential risk posed by the rapid expansion of wind energy developments across the world. Here, we provide the first microsatellite marker panel for the Nathusius' pipistrelle, a migratory bat species that is amongst the most frequently observed casualties at wind farms in Europe. By employing this panel to a four year dataset of over 400 individuals sampled along the Dutch coastline during the autumn migration period, we provide a first baseline estimate of current genetic diversity and address several unresolved questions regarding the population structure and migratory behavior of the species.

Population genetic structure

We find no evidence of population sub-structuring or deviation from Hardy-Weinberg equilibrium in our dataset, suggesting that all individuals that reside or migrate along the Dutch coastline belong to a single panmictic population. These observations are consistent with descriptions of long-distance male dispersal and establishment of mating territories along the migratory pathways (e.g. Pētersons 2004), which result in gene flow between populations from a wide summer catchment area. Based on ring recoveries and proposed migratory pathways (Russ 2022), this suggests that the entire Fennoscandian and Baltic region may effectively represent a single genetically unstructured population. Similar patterns of weak population structuring have been observed in two other European migratory bat species, *Pipistrellus pygmaeus* (Bryja et al. 2009) and *Nyctalus noctula* (Petit & Mayer 1999). However, in *N. noctula*, weak population structuring and limits to gene flow were observed in some populations, possibly caused by geographic barriers (Petit & Mayer 1999). Further sampling and

analysis of Nathusius' pipistrelle across its distribution range and along other migratory pathways are needed to evaluate whether similar patterns exist in this species.

Genetic diversity and trend

Overall, we observed a genetically diverse population, with an effective population size estimate in the hundreds of thousands that cannot be distinguished from a population of infinite size. Detecting population decline and genetic erosion in large populations is notoriously difficult, and simulations have shown that even substantial demographic declines may not be distinguished (Hoban et al. 2014). Nevertheless, the size and diversity of the genetic population being investigated here may in fact be favorable to detecting population declines, and we observed two indicators that may be indicative of such recent population decline.

First, we observed a consistent decline in allelic richness over the four sampled years. Microsatellite loci accrue variation (new alleles) over time through mutation, and as a result large populations may be highly polymorphic at such loci, with many of the alleles being present at very low frequencies. As such populations decline, these rare alleles may readily drift to extinction, resulting in a reduction in allelic richness, but little to no change in expected heterozygosity (Hoban et al. 2014). Such reductions in allelic richness have been observed empirically for abundant marine fish species (Pinsky & Palumbi 2014), where allelic richness was 12% lower on average in overfished populations. In fact, large populations may stand to lose disproportionately large amounts of allelic diversity, as illustrated by Allendorf et al. (2024), who calculated that a reduction of Baltic herring populations from 31 billion to 9 billion individuals could be expected to reduce the number of alleles in the population by approximately 70% if the population were to remain in drift-mutation equilibrium. While the Nathusius' pipistrelle

412 population present in Northern Europe almost certainly does not total in the billions, it
413 has likely accrued a substantial amount of genetic diversity over the course of several
414 centuries. Thus, while the difference in allelic richness observed here is still subtle
415 (2.5% between the first and last year) and is not statistically significant, it nevertheless
416 potentially represents a considerable decline in overall population size that deserves
417 further investigation.

418 Second, we observed a marked trend towards heterozygote excess in both
419 juvenile males and females. As above, during rapid population declines both allelic
420 diversity and heterozygosity decline, but at different rates, with allelic diversity declining
421 more rapidly (e.g. Hoban et al. 2014). This results in a transient period where the
422 observed number of alleles is lower than the number of alleles expected under
423 mutation-drift equilibrium (ie. a heterozygote excess; Cornuet & Luikart 1996). Thus,
424 the heterozygote excess observed here, while again statistically insignificant, may
425 point towards a recent population decline.

426 Despite being categorized as a species where direct risk factors are likely
427 impacting the population (e.g. Meinig et al. 2020), robust population trends using
428 traditional survey methods are rare. A TRIM-analysis conducted on box survey data
429 from North Rhine-Westphalia in Germany, suggests that the local population has
430 strongly declined since 2000 (Meinig et al. 2020). In acoustic surveys at offshore sites
431 in the North Sea, Lagerveld et al. (2023) found a significantly lower activity in 2020
432 than in the three years prior, but concluded that there was insufficient evidence to
433 definitively establish a decline. Therefore, the genetic indications of a potential decline
434 observed here are broadly concordant with those based on other survey
435 methodologies, but a broad-scale and long-term monitoring that corroborates these
436 results is needed.

437

438 **Mother-offspring guidance**

439 We found no evidence for mother-offspring pairs across a sample of thirty boxes where
440 both adult females and juvenile bats were present together (n = 93 total potential pairs).
441 Indeed, no parent-offspring pairs were recovered across the entire dataset, strongly
442 suggesting that offspring do not migrate together with their mothers in this species.
443 Baerwald & Barclay (2016) reached the same conclusion for two migratory bat species
444 in North America (*Lasiurus cinereus* and *Lasionycteris noctivagans*), based on genetic
445 samples taken from wind turbine fatalities. Instead, it appears plausible that Nathusius'
446 pipistrelles are born with a genetically pre-defined migratory vector, as observed in
447 many migratory songbirds (Berthold 2001), and use the Earth's magnetic field to
448 navigate (Holland et al. 2006; Lindecke et al. 2021) towards their goal.

449 Nevertheless, juvenile bats may (additionally) use cues from other conspecifics
450 during migration, such as their siblings or other colony members. We found one pair
451 of juvenile females, out of 43 juvenile pairs that were caught simultaneously from the
452 same bat box, related at the half-sib level. However, we cannot establish whether these
453 individuals are maternally or paternally related, and thus cannot evaluate whether they
454 may have migrated together. Moreover, while there is consensus that most mothers
455 give birth to two offspring (reviewed in Vierhaus 2004), it is unclear whether these
456 individuals are related at the full-sib or half-sib level, or a mix of both. Regardless, it
457 does not appear to be a widespread phenomenon, as the vast majority of juveniles
458 was not found with related conspecifics. The use of social cues from non-directly
459 related conspecifics would be impossible to detect genetically. Acoustic surveys have
460 shown a high concentration of Nathusius' pipistrelle activity along coastlines during the
461 migratory period (Ahlén et al. 2009, Ijäs et al. 2017), suggesting that juvenile bats could

potentially locate and use cues from unrelated conspecifics during migration at such landscape features. However, Baerwald & Barclay (2016) found no evidence that wind turbine fatalities originated from the same areas, using stable isotope analysis.

Conservation Implications and Recommendations

Assessing the threat posed by anthropogenic change to environments requires a fundamental understanding of the biology and population dynamics of the affected species. The establishment of a microsatellite panel for the Nathusius' pipistrelle has allowed us to contribute to several outstanding questions. For one, the lack of population sub-structuring in the individuals that migrate along the Dutch coastline means that the population can be managed as one entity. This is fortunate, as it means that methods such as acoustic monitoring and telemetry can be applied without caveats regarding the unknown population assignment of observed individuals.

Next, our findings, combined with those of previous studies, suggest that juvenile bats migrate according to an innate migratory vector, perhaps in combination with some social cues from unrelated conspecifics. The lack of mother-offspring guidance implies that juveniles will not immediately face higher mortality risk if their guide (mother) is killed along the migratory route. However, if migratory behavior is genetically pre-defined, juveniles may be highly susceptible to mortality at wind farms development placed along important landscape features (e.g. coastlines) in the vector direction that they instinctively follow.

Perhaps most importantly, our study highlights the feasibility of genetic methods as a monitoring tool, that may allow for inference regarding the population dynamics of a species that is otherwise very difficult to monitor accurately. Systematic genetic monitoring of trends in allelic richness and other diversity metrics, coupled with forward

genetic simulations of varying scenarios of population size and decline, could provide indispensable insights into the trajectory of the population. Similarly, analysis of historical samples (ie. collections of carcasses from wind-farm surveys, museum specimens), could provide valuable context regarding how genetic diversity has already changed. While such genetic diversity monitoring will undoubtedly remain comparatively crude, if declines are strong enough, they will provide irrefutable proof of population decline that can contribute to evidence-based action plans that strive for adequate species protection (e.g. more conservative curtailment regimes during migration). We hope that this work can act as a baseline reference of genetic diversity in the Nathusius' pipistrelle, and encourages further genetic monitoring of the species throughout its range.

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Statements and Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

The study was conceived by J.vS., R.J., D.D., J.S., and S.L.; Sample collection was coordinated by K.S., and performed by K.S., RJ, DD, J.P.C.B., B.N. and S.L.; Labwork and statistical analysis were performed by J.vS. and S.S.; Funding acquisition and project management were performed by J.vS., J.S. and S.L.; The first draft of the manuscript was written by J.vS., and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

Newly developed microsatellite loci were deposited in GenBank under Accession Nos. PQ641316- PQ641332. Complete sample information and genotypes are available in Supplementary Table S5.