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RESEARCH ARTICLE

Identification of conservation units in the European *Mergus merganser* based on nuclear and mitochondrial DNA markers

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Abstract The conservation status of small breeding areas of the Goosander (*Mergus merganser merganser*) in Central Europe is unclear. Geographic isolation of these areas suggests restricted gene flow to and from large North-European populations. On the other hand, migrating Goosanders from northern Europe join the Central European breeding population for wintering. To evaluate the conservation status of the small breeding areas we assessed the genetic structure of *M. merganser* populations in Europe by examining two nuclear marker systems (microsatellites and Single Nucleotide Polymorphisms, SNP) and mitochondrial (mtDNA) control region sequence variation for Goosanders in 11 sampling areas representing three of five distinct breeding areas and two subspecies (*M. m. merganser* and *M. m. americanus*). Overall population differentiation estimates including both subspecies were high, both based on mtDNA ($\Phi_{ST} = 0.899$; $P < 0.0001$) and nuclear markers ($\theta_{ST} = 0.219$; 95% CI 0.088–0.398, SNP and microsatellites combined). Within Europe, mtDNA revealed a strong overall ($\Phi_{ST} = 0.426$; $P < 0.0001$) and significant pairwise population differentiation between almost all comparisons. In contrast, both nuclear marker systems combined revealed only a small overall genetic differentiation ($\theta_{ST} = 0.022$; 95% CI 0.003–0.041). The strong genetic differentiation based on female-inherited mtDNA but not on biparentally inherited nuclear markers can be explained by sex-biased dispersal and strong female philopatry. Therefore, small breeding areas in Europe are endangered despite large male-mediated gene-flow, because when these populations decline, only males—but due to strong philopatry not females—can be efficiently supplemented by migration from the large North European populations. We therefore propose to manage the small breeding areas independently and to strengthen conservation efforts for this species in Central Europe.

Keywords Female philopatry · Microsatellite · mtDNA · Population structure · SNP

Introduction

The Goosander (*Mergus merganser*) has a holarctic distribution, with a wide breeding range across Eurasia and North America (Hagemeijer and Blair 1997). At present, three subspecies are recognised. *Mergus merganser comatus* occurs in Central Asia and *M. m. americanus* in North America. In Western Eurasia, the main breeding range of the nominate form *M. m. merganser* spreads from Iceland over Britain and Fennoscandia eastwards to northern Russia and Siberia (Hagemeijer and Blair 1997) (Fig. 1). In addition, the Goosander breeds in the region of the Alps (mainly Switzerland, and adjacent regions in France, Germany and Austria), and in the southern Balkan region (mainly Greece and Serbia-Montenegro). The small breeding populations in Iceland (100–300 pairs; BirdLife International 2004) and in

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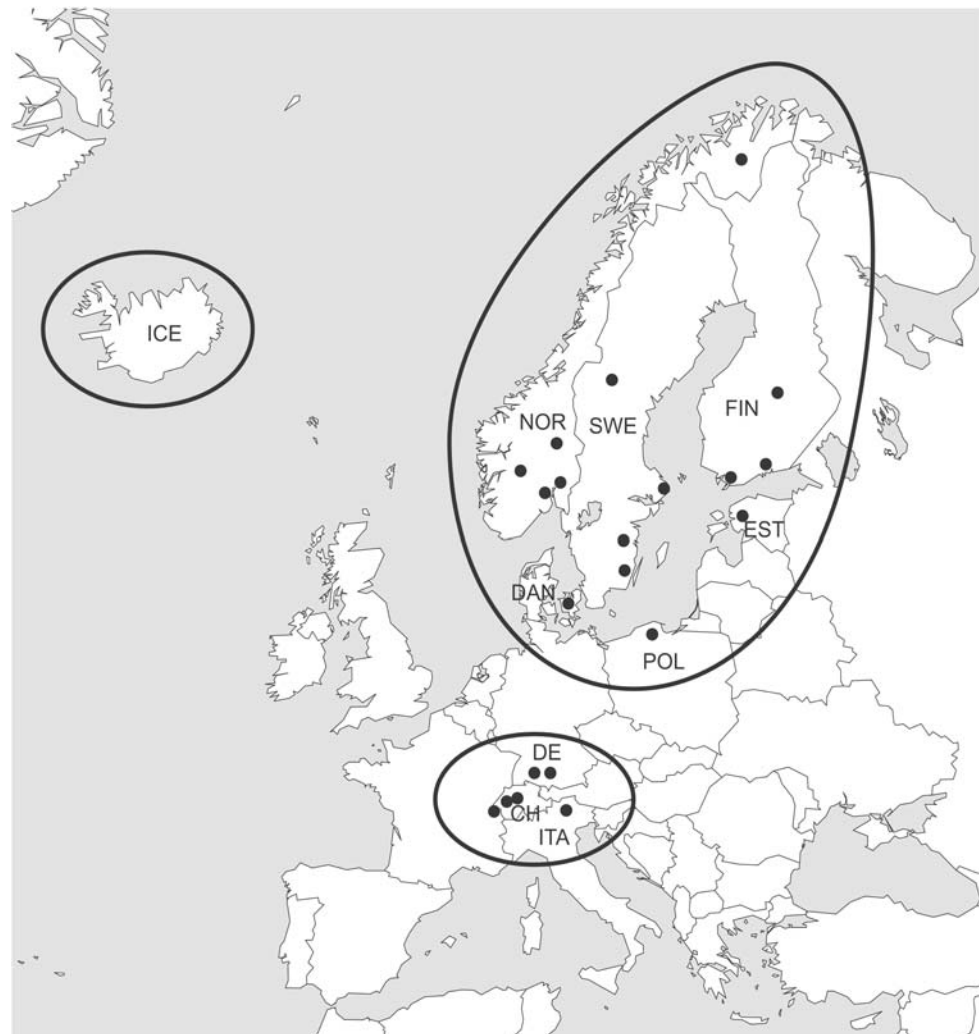

Fig. 1 Sampling sites in Europe for the three breeding areas Northern Europe (comprising NOR Norway, SWE Sweden, FIN Finland, EST Estonia, POL Poland, DAN Denmark), Alps (CH Switzerland, DE Germany, ITA Italy) and Iceland (ICE)

the Balkans (40–60 pairs; BirdLife International 2004) are considered isolated and non-migratory (Scott and Rose 1996). The degree of isolation of the British birds (2,900–3,600 pairs, BirdLife International 2004) and the birds from Central Europe (1,000–1,400 pairs, Keller and Gremaud 2003) is unclear. Although they have geographically distinct breeding areas these are within the wintering area of Fennoscandian birds (Scott and Rose 1996; Hofer and Marti 1988).

Overall, the Goosander is not endangered in Europe but the conservation status of the birds in the small breeding areas, especially in the region of the Alps, is unclear or controversial. In Switzerland, for example, where the majority of the pairs in the alpine breeding area occur, *M. merganser* is red-listed (category VU vulnerable, (Keller et al. 2001)) and hunting is prohibited. The same applies to the Bavarian birds (category 2, very endangered, Fünfstück et al. 2003). In the last decades, however, *M. merganser* has extended its breeding range in the alpine region and the number of breeding pairs has been increasing (Keller and Gremaud 2003). While the majority of both breeding and wintering Goosanders concentrate on large lakes, an increasing use of rivers has been observed, which has led to concerns among anglers who fear a negative effect on local fisheries. Thus, there exists an increasing pressure on re-evaluating the conservation status of *M. merganser* in Europe and especially in the region of the Alps, to determine whether certain breeding populations merit a separate management and a high priority for conservation.

To determine whether two populations need to be managed as distinct units an evaluation of their genetic exchangeability is required (Crandall et al. 2000). Genetic exchangeability is rejected if there is evidence of restricted gene flow between populations (Crandall et al. 2000). The geographic isolation of the breeding areas in the Alps, the Balkans, the British Isles and Iceland suggests a restricted gene flow among populations and thus genetic substructuring of European *M. merganser*. In addition, pairs commonly return to the female's natal site or prior nesting

area, suggesting a clear separation of at least the female breeders. However, like in many waterfowl species, pair formation occurs on the wintering grounds or during spring migration (Kalbe 1990). During this time, northern migratory birds join the resident breeding birds in, for example, the Alps and the British Isles. It is therefore also possible that considerable gene-flow occurs between migratory and resident breeding populations.

The objective of this study was to assess genetic exchangeability of and the gene flow among European *M. merganser* populations using two types of nuclear markers (microsatellites and single nucleotide polymorphisms, SNPs) and mitochondrial DNA (mtDNA) control region sequences. The combined results of all three types of genetic markers will allow the evaluation of the conservation status of the small *M. merganser* breeding populations in Europe.

Methods

Sample collection and DNA extraction

We distinguish between the terms breeding area and sampling area. The term breeding area refers to the five geographically distinct *M. merganser* breeding areas known in Europe; Iceland, British Isles, Northern Europe (i.e. Fennoscandia, Baltic States, Poland, northern Germany), the region of the Alps and the Balkan (Fig. 1). Because the exact boundaries of these breeding areas are uncertain, we based our analysis on sampling areas (i.e. geographically more narrowly defined areas where samples were collected). A total of 203 *M. m. merganser* and *M. m. americanus* samples were collected by over 30 volunteers in 11 such sampling areas (Fig. 1), two in Canada (New Foundland and Nova Scotia/New Brunswick) and nine in three of the five geographically distinct breeding areas: Iceland, Switzerland, Germany (where all samples were from Bavaria), Norway, Sweden, Denmark, Finland, Estonia and Poland (Fig. 1). One single sample came from Italy.

All *M. m. americanus* samples were collected in autumn, whereas the European samples were collected during the breeding season. Samples were collected from shot birds ($N = 34$), from females caught near their nest boxes and released after plucking three to five feathers ($N = 61$), and by visiting nest boxes and collecting feathers and egg skin samples ($N = 43$). Museum specimen feathers were available from Norway ($N = 23$) and Iceland ($N = 21$). In addition, we analyzed tissue ($N = 14$) and feather ($N = 7$) samples from birds shot or caught for ringing in Switzerland during winter time. These samples were not included in the population genetic analysis based on the breeding areas. During winter, migrating *M. merganser* from northern Europe join the Central European breeding population and it is thus likely that the samples collected during winter time belong to more than just one breeding area.

DNA was extracted using QIAamp® DNA Mini Kit (Qiagen) according to the manufacturer's instruction. From each Museum specimen two feathers were extracted independently as described in Gautschi et al. (2003).

Mt DNA control region amplification and sequencing

Amplification of a 1,047 bp fragment of the mtDNA control region (CR) was performed using primers L14990 (5′-AACATCTCCGCATGATGAAA-3′) (Kocher et al. 1989) and H16064 (5′-CTTCGATTTTTGGTTTACAA GACC-3′) (Sorenson et al. 1999). Additional primers MMCRL F (5′-GTGTTTGGCTATGTACGTCGTG-3′), MMCRL R (5′-TGGTGGAAGTAGTCCACAGATG-3′), MMCRR F (5′-ATCAATTGGGTTCACTCACCTC-3′) and MMCRR R (5′-GAGGAGTTGGGCTAAATGTTTG-3′) internal to the larger segment were designed to amplify shorter fragments of 514 bp (left domain: MMCRL primers) and 522 bp (right domain, MMCRR primers). For some ancient DNA samples it was not possible to amplify the two 500 bp fragments, and both CR-fragments had to be further divided into two smaller, overlapping fragments using the following primers; primers MMCRL RK (5′-TGGGGACA GTTGGTTGTTATG-3′) and MMCR F amplify a 280 bp fragment, primers MMCRL FK (5′-AGACCCCATCAAAT GAATGC-3′) and MMCR R amplify a 289 bp fragment, primers MMCRR RK (5′-ACTGAATGCGGACAAAGT GC-3′) and MMCR F amplify a 294 bp fragment, and primers MMCRR FK (5′-GTTGGCGTATATGGGGAA TC-3′) and MMCRR R amplify a 283 bp fragment.

DNA amplification reactions contained 10 ng of genomic DNA, 0.5 μM of each forward and reverse primer, 150 μM per dNTP, 0.5 U HotStarTaq™ DNA Polymerase (Qiagen) and 1 μl 10× reaction buffer (HotStarTaq™, Qiagen) containing Tris–Cl, KCl, $(NH_4)_2SO_4$ and a final concentration of 1.5 mM $MgCl_2$. We used the following thermotreatment on a PTC-225 machine (MJ Research, Inc.): 30 cycles with 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Before the first cycle, a prolonged denaturation step (95°C for 15 min) was included, and the last cycle was followed by an 8 min extension. PCR products were treated with ExoSAP-IT® (Amersham). Fragments were sequenced in both directions as described below and assembled, edited, and aligned with Sequence Navigator Software (Applied Biosystems).

Microsatellite genotyping

Microsatellite diversity was assessed in the sampling areas from which we had information from 10 and more

individuals (Table 1). We used eight autosomal microsatellite primers specifically developed for *M. merganser*. The primer sequences, the conditions for the amplification of each locus, and the methods for allele detection were as described in Gautschi and Koller (2005). DNA from non-invasively collected samples and museum specimens was amplified 3–8 times (i.e. multiple tube approach; Taberlet et al. 1999) to be able to detect allelic-drop out and the generation of false alleles. In addition, only samples for which at least five microsatellite loci could be typed unambiguously were used in the analysis.

Development and genotyping of SNP-markers

Total genomic DNA was isolated from tissue samples of two individuals using a standard phenol-chloroform extraction protocol (Sambrook et al. 1989). We digested 10 μg of the isolated DNA with 10 U each of *Alu*I, *Hae*III and *Rsa*I (New England Biolabs) for 3 h. Fragments of 200–700 bp were isolated with UltraPure™ Low Melting Point Agarose (Invitrogen) according to Sambrook et al. (1989), and were ligated into dephosphorylated pUC18 (pre-cut *Sma*I/BAP, Amersham Pharmacia). Following transformation of JM109 High Efficiency competent cells (Promega) and plating onto selective agar media containing X-Gal, IPTG and ampicillin, 73 recombinant clones were picked and plasmids were isolated using the QIAprep Spin Miniprep kit (Qiagen). Inserts were sequenced using M13 forward and reverse primers and the DYEnamic™ ET Terminator kit with Thermo Sequenase™ (Amersham Pharmacia). Sequences were analyzed on a MegaBACE 1000 DNA Analysis System (Amersham Pharmacia) and edited with Sequence Navigator Software (Applied Biosystems). Sequences were compared with GenBank to detect and avoid any coding regions. Primers were designed to preferably span the whole insert.

A total of 30 fragments were amplified in a panel of eight individuals. To avoid an ascertainment bias during SNP selection, we chose two random individuals each from four different sampling areas (i.e. Canada, Finland, Switzerland and Iceland). PCR amplifications were performed in a 10 μl reaction volume containing 20 ng of genomic DNA, 0.5 μM of each forward and reverse primer, 150 μM of each dNTP, 1× PCR buffer (Tris–Cl, KCl and $(NH_4)_2SO_4$, with a final concentration of 1.5 mM $MgCl_2$; Qiagen) and 0.5 units of HotStarTaq™ DNA Polymerase (Qiagen). We used the following thermotreatment on a PTC-225 machine (MJ Research): 30 cycles with 95°C for 30 s, locus specific annealing temperature (Table 2) for 30 s, and 72°C for 30 s. Before the first cycle, a prolonged denaturation step (95°C for 15 min) was included and the last cycle was followed by an 8 min extension.

PCR products were treated with ExoSAP-IT® (Amersham) to remove unconsumed dNTPs and primers, and fragments were sequenced in both directions as described above. Potential SNPs (i.e. overlapping electropherogram peaks, point mutations among individuals) were identified in 18 out of the 30 sequenced fragments. SNPs were then genotyped in the same panel of individuals by primer extension reaction (Sokolov 1990), in which screening primers, designed with a 3′ end immediately adjacent to the SNP (Table 3), undergo a one nucleotide extension by a fluorescently labelled ddNTP that corresponds to the SNP allele (Seddon et al. 2005). Reproducibility of the primers

Table 1 Sampling areas, sampling area identities (ID), and sample sizes for the analysis of the mtDNA (haplotype and AMOVA analysis) and analysis of the nuclear DNA (microsatellites and SNP), respectively

Sampling area	ID	mtDNA haplotype determination	mtDNA AMOVA	Microsatellite and SNP analysis
Canada (East Coast)	CAEC	14	14	14
Canada (New Foundland)	CANF	18	18	18
Denmark	DAN	3	–	–
Estonia	EST	3	–	–
Finland	FIN	37	37	39
Germany (Bavaria)	DE	9	9	–
Iceland	ICE	16	16	20
Italy	ITA	1	–	–
Norway	NOR	25	25	13
Poland	POL	7	7	–
Sweden	SWE	13	13	10
Switzerland (summer)	CHS	36	36	36
Switzerland (winter)	CHW	21	–	–
Total number of samples	–	203	175	150

Table 2 PCR primers used in *M. merganser* to amplify random DNA fragments for SNP analysis

Locus	Amplification primers for modern DNA samples 5′-3′	Size (bp)	Amplification primers for aDNA samples 5′-3′	Size (bp)	Multiplex	TA (°C)	GenBank Accession No.
SNP03	F: AGAGGGTGGTTTCACACTGG	421	F: AGAGGGTGGTTTCACACTGG	154	1	58	EU017495
	R: CGACTGACAAGACAGTGAATTATG		R: GGATCATCAAGTCCAACTCTTG				
SNP14	F: TTCTCCTGTCCCTGTGCATC	342	F: GCGGATTTCCGACTTCTTAG	152	1	58	EU017496
	R: CAGTGTAGCACAGCAACAATTC		R: AAACCATTTCCCTCCATTCC				
SNP19	F: ATGATGCATATGTAACTTGCTACC	319	F: ACACACAGCAAGTTGGATGC	141	1	58	EU017497
	R: GGACCCGATGATCTTTTGAG		R: TGTGCAAAGCCATTCAAAAC				
SNP25	F: TTCCTGGTATTTCAATCTGTGC	310	F: TTCCTGGTATTTCAATCTGTGC	150	1	58	EU017498
	R: CAATCCGACATGAAAAATCTCC		R: ACCAAAACATGAGTCGTCTGG				
SNP30_2	F: TGGGCAACCAAAGTTAAGG	320	F: AAAAGCAGCAGGCACTTCAC	135	2	58	EU017499
	R: TGGTCATCATTAACACACAGAGG		R: GGGTGCTTGGAGAAGGTCAC				
SNPC2_2	F: ATGCTAATCAAAGCGTTGTGTC	231	F: CTACCCCTGCCTGCCATAGT	173	3	58	EU017500
	R: CCCTTTGGCACCTATTCTCTG		R: CTCCAGTGTGCTGGAAGATG				
SNPC7	F: TTTCTCTTCCAGGATTTCTACCC	253	F: TTTCTCTTCCAGGATTTCTACCC	190	3	58	EU017501
	R: TCCAGGGAAGATATTTTCAGAATC		R: CTCCAGTGTGCTGGAAGATG				
SNPE7_2	F: CAG CCA AGG GTG AGT TAA GC	305	F: GTGGTTCTGAGCCGAGGTAG	130	2	58	EU017502
	R: TTC CCA CTG CAG TTT TGC TG		R: GCGCTAAAAACGAAAAGTGC				

Two pairs of primers are given per locus, which amplify fragments of different lengths for the analysis of modern and aDNA samples, respectively. TA = locus-specific annealing temperature

was tested by comparison with sequence data. Of the 18 fragments tested, 12 were genotyped successfully. However, four fragments had to be abandoned after screening a larger sample set, because the peaks could not be interpreted unambiguously in many samples. Thus in the end, eight fragments were amplified, and one SNP each was typed.

To assess SNP variation in the modern DNA samples, SNP loci were multiplexed (Table 2) and primer extension reactions were performed using the SNuPE kit (Amersham, Batley and Hayes 2003) as described in Seddon et al. (2005).

The same procedure was used for museum specimen and non-invasively collected DNA samples, with the following adaptations. Genetic diversity in low-quality DNA samples was assessed using primers amplifying fragments of 200 bp in length and shorter (Table 2), because the DNA was often degraded and only short fragments could be amplified. In addition, multiplex PCR amplification was performed with an increased number of cycles (40–45 cycles). As for microsatellite markers, SNP analysis was repeated for these low DNA quality samples to ensure reproducibility.

Table 3 *Mergus merganser* SNP primers used for genotyping by primer extension

Locus	Internal primer for primer extension 5′-3′	Direction	SNP
SNP03	GGTCTACCCAAAAGTTCAGA	R	C/T
SNP14	AAAGCACATTTTGTGGCACG	F	C/T
SNP19	GTTAGCATTTTCCATTACTTGTTCC	R	C/A
SNP25	ACTGAGAAAAGCCTGGTCCT	F	T/G
SNP30_2	ACTCCACACAGATCCCCAG	R	G/A
SNPC2_2	GGCTGTATCTGAAGTGCCGA	F	C/T
SNPC7	GCATGGTGTGCTGGTGCC	F	G/A
	ATCGCTTTGCCAGCAGGAG	R	C/T
SNPE7_2	GTTTTCTTCCAGACCTCCA	F	C/T

For SNPC7 two internal primers were tested and used successfully

Statistical analysis

Table 1 gives an overview of the samples per sampling area and the sample sizes used in the analysis of the mitochondrial and the nuclear DNA, respectively. We also determined the haplotypes of the 21 samples collected during winter in Switzerland. However, because the winter population consists of a mixture of breeding birds and winter guests, these samples were not included in the population genetic analyses.

The number of haplotypes (na), number of polymorphic sites (S), haplotype diversity (H $\pm$ SD) and nucleotide diversity ($\pi \pm$ SD) were estimated using DNASP (version 4.10.3) (Rozas and Rozas 1999). The hypothesis of selective neutrality of the control region fragment sequenced was tested using the D* test (Tajiama 1989).

An analysis of molecular variance (AMOVA) was carried out to assess the significance of genetic differentiation

between the two subspecies and among sampling areas within each subspecies using the program ARLEQUIN version 2.000 (Schneider et al. 2000). Included in the analyses were all sampling areas from which we had sequenced at least seven specimens (Table 1). Both haplotype frequencies and pair-wise differences among haplotypes were taken into account to estimate Φ_{ST}. The significance of Φ_{ST} estimates was tested by permutation of haplotypes among sampling areas among subspecies (1,000 permutations). Assuming a finite-island model, the effective female gene flow (N_em_f) between sampling areas was estimated using the following migration/drift equilibrium: $N_em_f = 1/2(1/\Phi_{ST} - 1)$.

Allelic diversity at the eight microsatellite loci was quantified overall and for each sampling area by the mean number of alleles per locus and the allelic richness. Observed and expected heterozygosities were determined independently for microsatellite and SNP markers. Statistical genotypic linkage disequilibria among loci and conformity with Hardy-Weinberg equilibrium at each locus, in each sampling area and for each marker type were tested with the FSTAT Software (Goudet 1999). We quantified the extent of genetic differentiation among sampling areas by computing Weir and Cockerham's (1984) estimator of F-statistics θ_{ST} using the FSTAT software package (Goudet 1999). θ_{ST} values from microsatellite and SNP data were first computed for each marker type independently and then for both marker sets combined. Significance of pairwise θ_{ST} was tested by Fisher's exact tests as implemented in GENEPOP (web version 3.4) (Raymond and Rousset 1995).

In order to test assignment of the Swiss winter samples to any of the breeding areas sampled in this study, we applied the Bayesian clustering approach implemented in STRUCTURE (version 2.0) (Falush et al. 2003).

Results

Mitochondrial DNA (mtDNA)

Based on an 888 bp fragment of the mtDNA control region, we observed a total of 42 haplotypes defined by 71 variable sites (Table 4). Excluding the three sites with gaps, overall haplotype diversity (H) and nucleotide diversity (π) among haplotypes were 0.918 ± 0.012) and 0.013 ($\pm$0.002), respectively. The haplotypes of the two subspecies differed at between 23 and 33 variable sites, with a mean of 28 nucleotide differences between the two subspecies (Table 4). Polymorphism was consistent with neutral expectations (Tajima's $D = -0.22$, $P > 0.10$).

The number of haplotypes per sampling area differed largely and ranged from 2 (Switzerland, Germany, Estonia, Denmark) to 12 (Norway) (Table 5). All sampling areas shared one or more haplotypes with at least one other area. However, none of the haplotypes found in the North-American subspecies *M. m. americanus* was found in Europe. Moreover, the isolated population from Iceland shared only one of its five detected haplotypes with other sampling areas (i.e. Finland and Sweden). Interestingly, Switzerland did not share any haplotypes with Bavaria, although both sampling areas belong to the same distinct breeding area (Fig. 1). However, the more common Swiss haplotype was also found in the single sample from Italy. The 21 samples collected in Switzerland during wintertime revealed haplotypes found in the breeding areas of Switzerland, Finland, Sweden, Norway, Denmark and Poland (Table 5) mirroring the mixture of Swiss breeding birds and winter guests during winter migration.

The AMOVA revealed a high degree of structuring with the largest proportion of the variation (81.19%; $P < 0.0001$) between the two subspecies and lower but also significant proportions of variation among sampling areas within subspecies, and within sampling areas (Table 6). Taking into account both haplotype frequencies and pairwise differences among haplotypes, population differentiation over all sampling areas (i.e. including the North American subspecies) was very strong ($\Phi_{ST} = 0.899$; $P < 0.0001$). Among the European sampling areas it was lower but still highly significant ($\Phi_{ST} = 0.426$; $P < 0.0001$). In addition, pairwise Φ_{ST}-values revealed significant population differentiation between almost all comparisons of sampling areas with the exception of Finland and Sweden where the Φ_{ST} value was negative, and between the two Canadian sampling areas (Table 7). Effective numbers of female migrants (N_em_f) ranged from 0.01 between Switzerland and New Foundland to 9.86 between the two Canadian sampling areas. Due to the negative Φ_{ST} value no N_em_f estimate is given between the samples of Finland and Sweden (Table 7).

Nuclear DNA

Mean number of alleles at eight microsatellite loci in the different sampling areas and subspecies ranged from 4.3 ($\pm$1.6) to 7.1 ($\pm$3.9), allelic richness from 3.8 ($\pm$1.5) to 4.7 ($\pm$2.0), and average observed heterozygosity values from 0.45 ($\pm$0.27) to 0.56 ($\pm$0.29). No diversity indices were significantly different among sampling areas and subspecies. Genetic variability at eight SNP loci is given in Table 8.

A significant departure from Hardy–Weinberg equilibrium after the sequential Bonferroni procedure (Rice 1989) was detected at the microsatellite loci MM04 and MM010 in the samples from New Foundland, but not in any of the other populations. No significant departure from Hardy–Weinberg equilibrium was observed within the sampling

Table 4 Polymorphic nucleotide sites defining 42 mitochondrial haplotypes resolved among 204 samples of *M. merganser*

Haplotype	Nucleotide position	Population ID
	11111111111111111111111112222222222222222222233355666777	
	34445666667777990011112222555566667788889011122333445555568800624014045	
	17898156783459067912890563569456715234672523446479260126905936406887058	
01	TTATTATCCCCAAGTCAATCCGATTTCTACAACTCTCCAACACAACAACTCC---CCATACGCCTCGTCCT	CHS, ITA
02	....T.....	CHS
03	......................G......T...........G.............................	DAN, DE
04	.....................AG.C................G......T..T.....G.............	DE
05	......................G..........C.......G.............................	EST
06	...................T..G..................G.......C.....................	SWE, FIN, EST
07	...........G......C...G..................G.............................	FIN, CHW
08	.............A........G..C..............TG.............................	NOR,SWE,FIN,CHW
09	.............A........G.................TG.............................	NOR, SWE, CHW
10	......................G..................G.............................	NOR,SWE,FIN,CHW
11	......................G..................G............................C	POL, DAN, CHW
12	...........G......C...G..........C.......G.............................	SWE, FIN
13	...........G..........G...........T......G..............T..............	FIN
14	...............T...T..G..................G.......C.....................	FIN
15	...........G..........G..................G.............................	ICE, SWE, FIN
16	C.....................G..................G.............................	FIN
17	...............T......G..................G.............................	FIN
18	...................T..G.......G..........G.......C.....................	FIN, SWE
19	....................T.G..................G.............................	SWE
20	......................G.................TG.........T.....G.............	SWE
21	......................G..................G.........T.....G.........C...	NOR
22	.............A........G..................G.............................	NOR
23	......................G..................G.....................T....TT.	NOR
24	......................G..................G.........T.....G.............	NOR
25	........T.............G..................G.............................	NOR
26	............G.........G..................G............................C	NOR
27	...................T..G..................G.............................	NOR
28	...........G..........G..................G.....................T.......	NOR
29	......................G..................G...............G.........C...	NOR
30	..G....A..T..AC..C.T....C...G.G......T.......T......AAC...........AC...	POL
31	..G....A..T..AC..C.T....C...G........T.......T......AAC...........AC...	POL
32	....AC...	POL
33	......CA..T......C.....CC.......T....TG........G.C..AAC...........AC...	ICE
34	.............A........G..................G.G..............C............	ICE
35	......CA..T......C......C.....G.T....TG........G.C..AAC............C...	ICE
36	......CA..T......C......C.....G.T....TG........G.C..AAC...........AC...	ICE
37	.C.CCG.T.T...ACTGC...A..C.TC.A....TCT..G....G.....T........GTA..C..C...	CAEC
38	.C.CCG...T...ACTGC...A..C.TC.A....TCT..G....G.....T........GTA..C..C...	CAEC, CANF
39	.C.CC....T...AC.GC...A..C..C.A....TCT..G....G.C........T...GTA..C..C...	CAEC
40	.C.CC....T...AC.GC...A..C..C.A....TCT..G..T.G.C............GTA..C..C...	CAEC
41	.C.CC....T...AC.GC...A..C....A....TCT..G....G.C........T...GTA..C..C...	CANF
42	.C.CC....T...AC.GC...A..C..C.A.G..TCT..G....G.C........T...GTA..C..C...	CAEC

Sequences are given in relation to haplotype 1, and dots indicate identity. For each haplotype the sampling area in which it was found is indicated (see Table 1 for abbreviations). The haplotype sequences are deposited on GenBank with accession No. EF675700–EF675741

areas at the eight SNP markers, nor was a significant linkage disequilibrium found between any pair of loci, independent of the marker type.

Both nuclear marker sets revealed a significant genetic differentiation among the seven sampling areas with an overall θ_{ST} of 0.087 (95% confidence intervals (CI) 0.061–0.115) and 0.483 (95% CI 0.134–0.782) for microsatellites and SNP markers, respectively. Both marker sets combined resulted in an overall θ_{ST} of 0.219 (95% CI 0.088–0.398). The differences between the two subspecies accounted for a large part of the overall genetic differentiation, as could be seen in pairwise θ_{ST} comparisons between the sampling areas from the two subspecies based on both data sets with values ranging from 0.362 to 0.424 (Table 9). The overall θ_{ST} of the European sampling areas was much lower, 0.022 (95% CI 0.003–0.041).

The small but significant θ_{ST} value within Europe can be attributed to the Icelandic sampling area, which was the only sample that differed significantly from the other European sampling areas ($\theta_{ST} = 0.039$–0.056, $P < 0.05$). Based on microsatellite markers only, the pairwise comparison between Iceland and Norway and Sweden led to a significant differentiation (data not shown). However, based on SNP markers only, these population differentiations were not

Table 5 Frequency of 42 mtDNA haplotypes in samples from 11 *M. m. merganser* sampling areas and two sampling areas from Canada (*M. m. americanus*)

Haplotype ID	Sampling area												
	$N = 36$ CHS	21 CHW	9 DE	3 EST	1 ITA	37 FIN	13 SWE	25 NOR	16 ICE	3 DAN	7 POL	14 CAEC	18 CANF
1	0.972	0.524	–	–	1	–	–	–	–	–	–	–	–
2	0.028	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	0.444	–	–	–	–	–	–	0.333	–	–	–
4	–	–	0.556	–	–	–	–	–	–	–	–	–	–
5	–	–	–	0.667	–	–	–	–	–	–	–	–	–
6	–	–	–	0.333	–	0.108	0.077	–	–	–	–	–	–
7	–	0.190	–	–	–	0.108	–	–	–	–	–	–	–
8	–	0.095	–	–	–	0.108	0.077	0.040	–	–	–	–	–
9	–	0.048	–	–	–	–	0.077	0.040	–	–	–	–	–
10	–	0.095	–	–	–	0.297	0.077	0.040	–	–	–	–	–
11	–	0.048	–	–	–	–	–	–	–	0.667	0.143	–	–
12	–	–	–	–	–	0.081	0.077	–	–	–	–	–	–
13	–	–	–	–	–	0.081	–	–	–	–	–	–	–
14	–	–	–	–	–	0.054	–	–	–	–	–	–	–
15	–	–	–	–	–	0.054	0.308	–	0.250	–	–	–	–
16	–	–	–	–	–	0.027	–	–	–	–	–	–	–
17	–	–	–	–	–	0.054	–	–	–	–	–	–	–
18	–	–	–	–	–	0.027	0.154	–	–	–	–	–	–
19	–	–	–	–	–	–	0.077	–	–	–	–	–	–
20	–	–	–	–	–	–	0.077	–	–	–	–	–	–
21	–	–	–	–	–	–	–	0.560	–	–	–	–	–
22	–	–	–	–	–	–	–	0.040	–	–	–	–	–
23	–	–	–	–	–	–	–	0.040	–	–	–	–	–
24	–	–	–	–	–	–	–	0.040	–	–	–	–	–
25	–	–	–	–	–	–	–	0.040	–	–	–	–	–
26	–	–	–	–	–	–	–	0.040	–	–	–	–	–
27	–	–	–	–	–	–	–	0.040	–	–	–	–	–
28	–	–	–	–	–	–	–	0.040	–	–	–	–	–
29	–	–	–	–	–	–	–	0.040	–	–	–	–	–
30	–	–	–	–	–	–	–	–	–	–	0.286	–	–
31	–	–	–	–	–	–	–	–	–	–	0.429	–	–
32	–	–	–	–	–	–	–	–	–	–	0.143	–	–
33	–	–	–	–	–	–	–	–	0.063	–	–	–	–
34	–	–	–	–	–	–	–	–	0.562	–	–	–	–
35	–	–	–	–	–	–	–	–	0.063	–	–	–	–
36	–	–	–	–	–	–	–	–	0.063	–	–	–	–
37	–	–	–	–	–	–	–	–	–	–	–	0.214	–
38	–	–	–	–	–	–	–	–	–	–	–	0.429	0.833
39	–	–	–	–	–	–	–	–	–	–	–	0.071	–
40	–	–	–	–	–	–	–	–	–	–	–	0.071	–
41	–	–	–	–	–	–	–	–	–	–	–	–	0.167
42	–	–	–	–	–	–	–	–	–	–	–	0.214	–

Haplotypes found in the samples collected in Switzerland during winter time are given in the column CHW. N = Number of samples, Haplotype ID = Haplotype identity

Table 6 Analysis of molecular variance (AMOVA) based on mtDNA control region sequence data for *M. merganser* grouped into sub-species

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
Among groups (subspecies)	1	631.244	11.61126*	81.19
Among populations within groups	7	173.630	1.23977*	8.67
Within populations	166	240.726	1.45016*	10.14
Total	174	1045.600	14.30119	

Hierarchy levels that contribute significant amounts to the overall variance are highlighted with * ($P < 0.001$)

Table 7 Pairwise Φ_{ST} (lower diagonal) and absolute number of female migrants per generation (upper diagonal) among nine *M. merganser* populations based on mtDNA control region sequences

	CHS	DE	FIN	SWE	NOR	POL	ICE	CANF	CAEC
CHS	–	0.09	0.32	0.18	0.18	0.08	0.43	0.01	0.02
DE	0.85	–	0.78	0.97	1.07	0.27	1.20	0.05	0.07
FIN	0.61	0.39	–	Inf	1.08	0.18	1.58	0.05	0.06
SWE	0.73	0.34	-0.02^{NS}	–	1.32	0.26	2.57	0.05	0.07
NOR	0.74	0.32	0.32	0.28	–	0.20	1.07	0.05	0.06
POL	0.87	0.65	0.73	0.65	0.72	–	0.59	0.07	0.11
ICE	0.54	0.29	0.24	0.16	0.32	0.46	–	0.10	0.12
CANF	0.97	0.91	0.91	0.91	0.92	0.87	0.84	–	9.86
CAEC	0.96	0.87	0.89	0.88	0.89	0.82	0.80	0.05^{NS}	–

Non-significant population differentiations are highlighted with (NS)

Table 8 Genetic variability at eight SNP loci in seven *M. merganser* sampling areas

Population	Locus																Observed heterozygosity	Nei expected heterozygosity
	SNP03		SNP14		SNP19		SNP25		SNP30		SNPC2		SNPC7		SNPE7			
	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2		
CHS	0.86	0.14	0.91	0.09	0.03	0.97	0.17	0.83	0.62	0.38	0.76	0.24	1.00	0.00	0.00	1.00	0.16 (±0.18)	0.20 (±0.18)
FIN	0.86	0.14	0.92	0.08	0.03	0.97	0.05	0.95	0.64	0.36	0.82	0.18	1.00	0.00	0.00	1.00	0.15 (±0.16)	0.16 (±0.16)
SWE	0.78	0.22	0.80	0.20	0.00	1.00	0.09	0.91	0.83	0.17	0.73	0.27	1.00	0.00	0.00	1.00	0.14 (±0.13)	0.19 (±0.17)
N0R	1.00	0.00	1.00	0.00	0.00	1.00	0.05	0.95	0.73	0.27	0.75	0.25	1.00	0.00	0.00	1.00	0.14 (±0.24)	0.11 (±0.14)
ICE	1.00	0.00	1.00	0.00	0.00	1.00	0.05	0.95	0.55	0.45	1.00	0.00	1.00	0.00	0.00	1.00	0.09 (±0.20)	0.08 (±0.18)
CANF	1.00	0.00	0.94	0.06	1.00	0.00	1.00	0.00	0.56	0.44	0.75	0.25	0.64	0.36	1.00	0.00	0.19 (±0.24)	0.18 (±0.22)
CAEC	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	0.68	0.32	0.64	0.36	0.54	0.46	1.00	0.00	0.20 (±0.28)	0.17 (±0.24)

A1 gives the frequency for allele 1, A2 the frequency of allele 2. The two frequencies add up to a frequency of 1 for the bi-allelic markers used in this study

significant, which was mirrored in the results from the combined data set, where, after the Bonferroni correction, the comparison between the samples of Iceland and Norway was not significant (Table 9).

Individual assignment tests of the Swiss winter samples based on the Bayesian clustering approach utilised by STRUCTURE failed to provide any meaningful estimates, probably due to the low levels of genetic differentiation among the breeding areas.

Discussion

Population structure

Our analysis revealed a significant genetic differentiation of the two subspecies *M. m. merganser* and *M. m. americanus* based on both nuclear and mtDNA markers. The genetic distinctiveness of the two subspecies was further underlined by the fact that the subspecies did not share a

Table 9 Pairwise θ_{ST} (lower diagonal) based on the combined SNP and microsatellite data set

	CANF	CAEC	CHS	FIN	ICE	NOR	SWE
CANF	–	NS	***	***	***	***	***
CAEC	0.0027	–	***	***	***	***	***
CHS	0.3626	0.3658	–	NS	***	NS	NS
FIN	0.3921	0.3962	0.0090	–	***	NS	NS
ICE	0.4190	0.4243	0.0558	0.0334	–	(*)	*
NOR	0.3789	0.3858	−0.0012	0.0079	0.0269	–	NS
SWE	0.3802	0.3874	0.0010	−0.0043	0.0389	−0.0102	–

Values were tested for significance (upper diagonal) by permutation of genotypes among populations. Significance was corrected by the sequential Bonferroni technique. The significance level after Bonferroni correction is 0.0024

NS, not significant

(*) Significant if comparison is based on microsatellite data only

single mtDNA haplotype. Within Europe, pairwise population comparisons revealed that only the Icelandic samples differed significantly from the other sampling areas based on both marker types, supporting the assumption that this geographically isolated breeding population is also isolated genetically. All other comparisons based on nuclear loci failed to reveal a significant genetic differentiation of the European sampling areas, suggesting that Goosanders in Europe belong to one large, panmictic breeding population. In contrast, based on mtDNA control region sequences, a significant genetic structuring over all European sampling areas was detected ($\Phi_{ST} = 0.426$; $P < 0.0001$) and apart from Finland and Sweden, all pairwise comparisons revealed highly significant population differentiations (Table 7).

Contrasting levels of genetic differentiation among populations based on nuclear and mitochondrial loci are detected in many species (e.g. Johnson et al. 2003; Chappell et al. 2004; Gay et al. 2004; Brown et al. 2005; Jones et al. 2005). There are several explanations for this phenomenon. First of all, structure indices estimated with nuclear and mitochondrial markers are not comparable directly, because mitochondrial gene flow only occurs due to female dispersal, whereas nuclear gene flow is equally affected by male and female dispersal. In addition, because the mitochondrial markers are uniparentally inherited and haploid, their effective population size is four times smaller than the one of the nuclear markers. Mitochondrial markers are therefore expected to reach equilibrium more rapidly than nuclear markers (Friesen et al. 1996; Edwards 1997). Thus, different genetic structures for mitochondrial and nuclear DNA events may be seen if mitochondrial but not yet nuclear markers have reached equilibrium (Hey and Harris 1999).

If we assume that none of the two factors mentioned above affected the *M. merganser* population structure in Europe (i.e. that the populations are at equilibrium and without sex-biased dispersal) the observed mitochondrial Φ_{ST} of 0.426 for the European sampling area would be equivalent to a theoretical nuclear θ_{ST} of 0.157 (Crochet 2000). However, the observed overall θ_{ST} value for nuclear markers in Europe is six to seven times lower (i.e. $\theta_{ST} = 0.022$ for both marker systems combined, $\theta_{ST} = 0.026$ and 0.009 for microsatellites and SNPs, respectively). Therefore, the difference between the two estimates has to be influenced by more than just the difference in the effective population size of the two marker systems used. Either the populations are in a non-equilibrium state and/or the difference may be explained by sex-biased dispersal.

If the strong mtDNA structure detected among the sampling areas and the lack of structure detected at the nuclear markers reflects a non-equilibrium state, the only possible explanation would be that gene flow among sampling areas had decreased recently and that nuclear allelic frequencies have not yet diverged accordingly (Friesen et al. 1996). However, the lack of shared mtDNA haplotypes and the number of mtDNA nucleotide differences between for example the Swiss samples compared to samples from other analyzed areas seem to support a long-term rather than a recent separation at least of this sampling area.

Thus, the more likely explanation for the difference in population structure between mtDNA and nuclear markers lies in the consequences of sex-biased dispersal. In fact, unlike the prevalent pattern of male-biased philopatry in other avian species, female Anatidae display a strong natal and breeding site fidelity (Rohwer and Anderson 1988). A strong female philopatry in *M. merganser* is also supported by ringing recoveries and nest box visits in subsequent years (Wernham et al. 2002; Swiss Ornithological Institute, unpublished data).

Difference of θ_{ST} estimates from SNP and microsatellite data

Overall θ_{ST} values showed large differences depending on which of the two nuclear marker systems the estimate was

based on. Including both subspecies, overall θ_{ST} estimates were 0.087 and 0.483 for microsatellite and SNP markers, respectively. Including only the European sampling areas, pairwise θ_{ST} based on eight microsatellite markers showed a significant genetic differentiation between Iceland and Northern Europe (i.e. Sweden and Norway) but no significant differentiation could be observed based on the equivalent number of SNP markers. Possibly, size homoplasy of microsatellite markers plays a role on time scales relevant to the subspecies splitting in *M. merganser* (Viard et al. 1998; van Oppen et al. 2000). Thus, microsatellite markers likely underestimate the genetic differentiation of the two Goosander subspecies. On the other hand, the significant population differentiation between Iceland and Northern Europe based on microsatellites, but not based on SNP markers, demonstrate the lower resolution of the bi-allelic SNPs in comparison to the multi-allelic microsatellite markers in studies of closely related populations. The lower information content of the SNP markers makes it necessary to use more SNP markers to reach the same resolution as microsatellite markers (Seddon et al. 2005).

Low mtDNA variability in Switzerland

Samples from birds breeding in Switzerland showed a surprisingly low mtDNA variability with a very common haplotype identified in 35 samples and a second haplotype identified in a single individual only. Other sampling areas also revealed two haplotypes but in all of them fewer individuals were sampled and their haplotype frequency was more balanced (Table 5). Despite the low mtDNA variability, the variability at the eight microsatellite markers of the Swiss breeding area was high (mean number of alleles 7.1 (±3.9), allelic richness 4.6 (±2.0), and mean Ho 0.54 (±0.26)). Only the 39 samples from Finland revealed slightly, but not significantly, larger microsatellite variability indexes (data not shown). These results suggest, that the current Swiss breeding population descended from only a few females. According to published records the first breeding Goosanders in Switzerland were observed around the middle of the 19th century on different lakes in the west, from where they spread towards the north-east in the 20th century (Keller and Gremaud 2003). Thus, the reduced variability could be explained by a founder effect.

The large variability at nuclear markers suggests that male-mediated gene flow among breeding areas over the last generations was high and compensated possible variability-deficiencies at nuclear markers caused by the founder effect. On the other hand, female-mediate gene flow to and from Switzerland is very low and almost inexistent.

Composition of Swiss winter population

The haplotype distribution of the samples collected in Switzerland during winter, confirm the observations that North European *M. merganser* migrate to Switzerland during winter (Hofer and Marti 1988). Based on census data, Keller and Gremaud (2003) estimated the proportion of Northern European winter guests to be around 50% or lower. In accordance with their estimate, about 52% of the 21 *M. merganser* samples collected in Switzerland in January and February 2002 revealed the distinct Swiss haplotype, whereas the haplotypes of the other individuals were found in the Northern European breeding populations. Due to low levels of genetic differentiation at both nuclear marker systems, the Swiss winter samples could not be unambiguously assigned to any of the sampled breeding populations.

Heterogeneity of the Central European breeding area

The Central European breeding area is thought to consist of one contiguous population distributed from France (Savoie), Switzerland and Bavaria to Austria (Scott and Rose 1996). Data on distribution and population trend, however, showed two centres of occurrence in the region of the Alps, one in Switzerland/France and the second in Bavaria/Austria. These centres are separated by a rather narrow gap (Keller and Gremaund 2003). In this study we had access to samples from Switzerland and a few samples from Bavaria only. The two sampling areas analyzed did not share a single mtDNA haplotype. In addition, the haplotypes found at these locations differed at as many as three to eight nucleotide positions. This supports the finding that the breeding area of *M. merganser* in the Alpine region may have to be further divided into smaller units. However, the very small sample size from Bavaria does not allow us to draw a definite conclusion and more samples need to be collected from Bavaria but also from France, Austria and Northern Italy, to be able to revise the boundaries within the Central European Breeding area. In addition, samples from the Balkan and the British Isles should be collected to be able to determine the status of the other geographically isolated breeding areas.

Conservation of *M. merganser* in Europe

Our results based on nuclear and mtDNA markers show that the geographically isolated *M. merganser* population of Iceland is also genetically distinct. It therefore needs to be treated as a separate management unit. With an estimated number of breeding pairs between 100 and 300, this

sedentary population is small and thus vulnerable to demographic and genetic risks.

Whereas male-mediated gene flow among the other investigated European sampling areas was strong and no genetic differentiation based on nuclear markers could be detected, our mtDNA results show a very strong fidelity of *M. merganser* females to their natal site with very low numbers of effective female migrants among sampling areas. Population sizes in Northern Europe are large with between 36,000 and 60,000 breeding pairs. However, the Alpine breeding populations are small with an estimated number of 620–870 breeding pairs in Switzerland and adjacent areas in France (Keller and Gremaud 2003), only 250–290 pairs in Bavaria (Von Lossow and Fünftsück 2003) and 140–200 pairs in Austria (Keller and Gremaud 2003). Small populations are more vulnerable to extinction than larger ones (Newmark 1995; Belovsky et al. 1999; Breininger et al. 1999), because survival is affected by several stochastic factors all of which increase in importance with decreasing population size (Matthies et al. 2004). Apart from environmental and demographic stochasticity, genetic uncertainty, the changes in genetic make-up due to genetic drift, inbreeding or founder effects may alter the survival and reproductive probabilities of individuals (Shaffer 1981;1987). In the present study, the potential genetic risks of the investigated small breeding populations in Central Europe are compensated by the strong male-mediated genetic exchange with the large populations in the North. However, the very low levels of female-mediated gene flow imply a considerable demographic independence of the small sampling areas (Avise 1995). If breeding populations in the Alps suffered from a severe population decline or even from extinction, it is very unlikely that they would recover or be re-established in the short term via recruitment of nonindigenous females. The *M. merganser* breeding populations in the Alps are therefore not exchangeable with the large Northern European populations. In addition, they are demographically small and thus have a high extinction risk. We therefore propose to manage the small breeding areas in the Alps independently from the large North European populations and suggest, to strengthen the conservation efforts for this species in the geographically isolated breeding areas of Central Europe.

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