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14.04.08

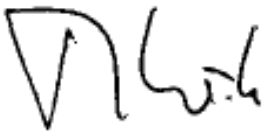
Comments to criticism by M. Ruokonen

Dear Bert Lenten

Dr Ruokonen had sent me her criticism in March. My coworkers and myself have studied the comments. We can agree with some arguments but disagree with others. We have prepared our own comments by now, which I attach to this letter. We hope that our letter will help to understand the intentions and results of our study.

We have not found an argument in the correspondence by M. Ruokonen and M. Osora, which would invalidate our scientific finding that the breeding stock of LWFG in Germany I suitable for a breeding and reintroduction program.

Best regards



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03.04.08

3-4-08

Dear Mrs Ruokonen

we have read your comments with interests; we can agree with a number of your arguments but by far not all. Therefore, we would like to provide comments on your criticism.

By going through the data, we have made two changes:

1. we had included 2 old LWFG samples from a Zoo, which is not participating in the breeding program, both birds showed hybrid origin. We have removed the 2 birds, because they are not relevant for the Zwerggans-Projekt. Therefore, the number of hybrids is 6 instead of 8
2. in the network we had excluded birds with autapomorphic haploytes (i.e. present in a single bird); we now have included these birds. This increases the number of haplotypes but does not alter not the conclusions.

Your criticism is highlighted in yellow…

Mitochondrial DNA

In the Table below are the mtDNA haplotype frequencies from the Table 3 in Pedall *et al.* reorganized by the species and populations. The first striking observation is that haplotype "LWFG1" found in both lesser and greater white-fronted geese is present only in the captive population of the lesser white-fronted goose. The fact that haplotype LWFG1 does not exist in the wild lesser white-fronted goose population strongly suggests that its presence in the captive lesser white-fronted goose stock is due to hybridization in captivity, and this is not clearly enough stated in the manuscript.

Comment: As can been seen from Fig.3, we do have wild Russian LWFG, which fall in the same lineage as LWFG1. The German captive LWFG studied came from 4 breeding stations in East Germany. Unfortunately we do not know, where the German LWFG originally came from. Because the former East Germany had excellent relationships with the former Soviet Union, it is very likely that the German LWFG came from somewhere in Russia. As we have different breeding flocks, we can safely assume that the birds came from several places in Russia. Thus we would expect, that the captive LWFG show a diversity of haplotypes. In contrast, the Russian wild birds came from a single locality near the Ural; it would have been very unlikely that these birds have the same haplotype as the captive birds, which probably came from several places. We also have theoretical problems with your statement "its presence in the captive lesser white-fronted goose stock is due to hybridization in captivity" . MtDNA is inherited maternally and shows no recombination. If our LWFG would be hybrids, than a hybridizing maternal line must be present. As clearly shown by the results; all other goose species (except lineage I of GWFG) show different DNA sequences; therefore a hybridization with them can be ruled out.

Table. Haplotype frequencies from Pedall *et al*. Table 3 listed according to species and populations.

	greater white-fronted goose		**lesser white-fronted goose**		**bean goose**	**greylag goose**
	Russia	**Germany**	**captive**	**Russia**	**Russia**	**Germany**
GWFG	12	-	-	-	-	-
LWFG1	-	45	42	-	-	-
LWFG2	-	-	2	-	-	-
LWFG3	-	-	10	-	-	-
LWFG4	-	-	10	-	-	-
LWFG6	-	-	2	-	-	-
LWFG5	-	-	-	7	-	-
LWFG7	-	-	-	4	-	-
LWFG8	-	-	-	2	-	-
LWFG9	-	-	-	4	-	-
LWFG10	-	-	-	2	-	-
LWFG11	-	-	-	2	-	-
BG	-	-	-	-	7	-
GLG	-	-	-	-	-	5
N	12	45	66	21	6	5

The second observation is even more striking: the captive and wild populations of the lesser white-fronted goose do not have a single mtDNA haplotype in common. In the captive population haplotypes LWFG1-4 and LWFG6 are found, whereas in the wild population haplotypes LWFG5 and LWFG7-11 are present. This is a very strange finding and the explanation is not obvious. Even if the sample size for the wild lesser white-fronted goose population is small, it is typical that common haplotypes are sampled with a greater probability than the rare ones, and the same fact applies to the captive populations as well. The probability that the captive population carries five lesser white-fronted goose haplotypes that are currently extinct in the wild population is extremely small, especially as it is known from previous work (Ruokonen *et al*. 2004) that there are two very common haplotypes (found in 64% of the individuals) present in the wild population. Possibly, this calls for reassessment of the methodological part of the work

Comment: I repeat my explanation from the previous comment. The German captive LWFG studied came from 4 breeding stations in East Germany. Unfortunately we do not know, where the German LWFG originally came from. Because the former East Germany had excellent relationships with the former Soviet Union, it is very likely that the German LWFG came from somewhere in Russia. As we have different breeding flocks, we can safely assume that the birds came from several places in Russia. Thus we would expect, that the captive LWFG show a diversity of haplotypes. In contrast, the Russian wild birds came from a single locality near the Ural; it would have been very unlikely that these birds have the same haplotype as the captive birds, which probably came from several places.

Setting the reason aside, the finding has consequences for the interpretation of the results. The

By the way: we had asked you during the project to share DNA samples from the Russian populations that you have studied. You had declined to do so. If you had provided your samples our analysis would be much better as regards to the geographical origin of the German LWFG.

purpose here would be to examine the genetic composition of the captive stocks based on the data obtained from the wild population as a reference sample. So, now the results tell that in the captive population there are four unknown haplotypes (LWFG2-4 and LWFG6) and one haplotype (LWFG1) in common with the greater white-fronted goose, the latter of which could suggest that 64% of the German captive lesser white-fronted geese have a hybrid origin. Also, as seen from the Fig. 3 in Pedall *et al*., the species do not cluster into monophyletic groups and e.g. the bean goose and the greylag goose are more closely related to the “lineage

II" than the lineage I and II are to each other suggesting that not enough resolution has been obtained with this marker. Therefore, it is impossible to say, or even to guess, based on the tree topology, to which species some of the haplotypes belong.

Comment: The cyt b data show a high degree of resolution; the main problem is that our coverage of LWFG and GWFG from the different Russian populations is extremely small. You should not forget however, that our task was not to find out from which Russian population the German LWFG came from, but to analyse for hybrids with other goose species.

The haplotype LWFG1 is shared with one lineage of GWFG. This is probably due to hybridization or due to a common history and recent speciation, which is clearly stated in the manuscript. The microsatellite analysis shows that very few captive LWFG carry GWFG alleles.

Nuclear DNA

In the results for the assignment test (program Structure) the authors do not state the findings clearly enough. When K=3, the groups correspond to 1) captive lesser, 2) wild lesser and 3) greater white-fronted goose + greylag goose, and when K=4, the groups are 1) captive lesser, 2) wild lesser, 3) greater white-fronted goose and 4) greylag goose. So, in both analyses, the program suggests that the captive and wild lesser white-fronted goose belong to different groups, which tells that they are differentiated from each other. A list of alleles shared by or private to the species/populations would have helped to evaluate the performance of Structure analysis.

Comment: In the assignment analysis with k = 2–3 captive LWFG and wild LWFG are clustered always in the same group. When k = 4, the groups consists of captive LWFG together with wild Russian LWFG (green colour in Fig. 6), GLG and GWFG. With k=4, the captive LWFG show alleles (red colour in Fig. 6) shared only with the wild LWFG; these alleles are not present neither in GLG nor in GWFG. Now, the proportion of individuals carrying those alleles by wild LWFG is lower compared to that one in captive LWFG; this difference may be a result of small sample size of wild LWFG (from a single origin, as mentioned above) or captivity.

Six individuals share alleles with GWFG or GLG (in contrast to the captive LWFG in Finland, in which we detected many hybrids with GLG!). These individuals will be removed from the reintroduction program.

Concerning the six putative hybrids found, it would have been essential to know which mtDNA haplotypes these individuals carried. This could have given an additional viewpoint for the analysis and especially for the conclusions. If the German captive lessers carry greater white-fronted goose nuclear alleles, there probably is also heterospecific mtDNA in the captive population.

The haplotypes of the hybrids are now mentioned in Table 7 together with the assignment probabilities.

Implications

The results of Pedall *et al*. do not differ from previous results in such a way that the common decision to not to use the present old captive stocks for reintroduction/population supplementation should be reconsidered. The German stocks were shown to include hybrids, but the manuscript is lacking an effort to try to clarify the situation in depth (e.g. how many captive stocks were sampled and how many of them were affected?). However, it seems that the hybrids come from different farms (M. Wink, pers. comm. to M. Osara), and this implies that there are probably also other birds affected, as the hybrids do not reproduce by themselves. This means that after removing the hybrids found, the captive stock can not be considered pure, contrary to the conclusion by Pedall *et al*.

Comment: Our curator M. Wolff from Cottbus Zoo did not take samples from all German LWFG, but concentrated on 4 breeding flocks in East Germany. It was not the intention to cover every single bird in captivity. Our project is a practical one; Aktion Zwerggans wants to establish a group of captive LWFG, that is genetically clean and large enough for a breeding program. We are convinced that this aim was achieved.

this implies that there are probably also other birds affected, as the hybrids do not reproduce by themselves : this is true, 4 of the 6 hybrids came from Cottbus Zoo,

Other comments:

Introduction: The lesser white-fronted goose has never been wintering in Kazakhstan, France or Hungary.

This sentence has been rephrased in the introduction.

Introduction: references missing for hybridization in the wild, as well as for the “old migration route” leading to Germany.

References for hybridization in the wild have been included in the manuscript.

Fig. 1 comes out of the blue, is not explained in the material and methods.
Fig. 1 is already explained in methodology.

Table 3 contains errors, a lot (e.g. nucleotide positions that do not seem to vary or contain only one of the nucleotides and dots: 16, 132, 162, 339, 492, 755, 763). Also, assigning haplotypes based on heteroplasmic nucleotide positions should be justified and the reasoning used should be explained: there are no simple rules for this. One could say that there are only seven "LWFG" haplotypes instead of 11.

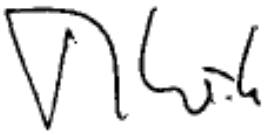
Table 3 was already corrected. The assignment of haplotypes is according to median-joining network algorithms (see Bandelt et al. 1999).

3.2. Positive Fis values: most likely due to Wahlund effect, considering the sampling strategy.
The Wahlund effect is now mentioned in the manuscript.

We have made changes in our original manuscript to improve clarity, as your comments made it clear, that we did not write down facts which were obvious for us.

Therefore, we would like to thank you for your comments.

Best regards



Prof. Dr. Michael Wink