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Recommendations for a reintroduction program of Lesser white front geese (LWFG) *Anser erythropus*: A genetic perspective

The critical status of LWFG populations in the World has been well documented. In order to prevent the extinction of the species special projects are definitely needed, among them reintroduction projects. The planned breeding program of "Aktion Zwerggans" coupled with guided migration to safe wintering grounds at the Niederrhein is certainly a valuable approach. Because its technical feasibility has been successfully demonstrated already, this project should be carried out if the genetic prerequisites are fulfilled.

A requirement for all breeding and reintroduction programs concerns the **genetic makeup of the founder animals**. The genetic makeup of breeding pairs should be comparable with that of wild populations. Special care needs to be taken, that breeding stock should be genetically pure and must not contain any hybrids between related species.

Since the goose species of the genus *Anser* are evolutionary young and thus closely related (Ruokonen et al., 2000a; this study), fertile hybrids are possible between some of them. Hybridisation can occur in captivity if members of different species are kept in the same enclosure. Hybridisation can also take place under natural conditions if an adult goose settles in a breeding colony of a related species.

Hybridisation is not a common phenomenon in LWFG but has been observed in LWFG, both in captive and wild birds. In captivity, hybrids are known between *A. anser* and *A. albifrons* (s. publications by Ruokonen et al 2000a,b, 2004). Wild hybrids have been detected between LWFG and *A. albifrons* (1 bird in the Amsterdam Zoo collection; Voous and Wattel 1967)

Can we guarantee genetic purity in LWFG?

Modern genetics provide powerful tools to analyse the genetic makeup of any goose species; these methods do not provide a 100% solutions but are much better than the methods we had

in the past. In the context of LWFG the following methods are useful and are employed in my laboratory:

1. **Sequence analysis of mitochondrial marker genes (mtDNA)**

Each goose species can be distinguished be a unique sequence of mtDNA (such a D-loop region (see Ruokonen et al. 2004; cytochrome b or ND2). Since mtDNA is inherited maternally, such an analysis can only reveal potential hybrids in the genetic lineage of the mother.

Using sequences of cyt b and ND2, my laboratory has found that the German breeding stock of LWFG has only few birds that are maternally of hybrid origin (less than 15%); most birds would fulfil the criterion of genetic purity, as far as the maternal lineage is concerned. As we have not yet analysed the Scandinavian birds, we cannot give an estimate for these birds. M. Ruokonen et al. (2000b) had suggested a higher level of hybridisation. We can easily corroborate earlier assumption, if we had the respective DNA samples.

2. **Analysis of the paternal lineage by genomic fingerprinting and microsatellite analysis**

Since sequences of the nuclear genome develop much slower than those of mtDNA, sequence data do not help to study the paternal lineage. Part of the genome shows a high degree of variability: short tandem repeats (STR) or microsatellites are common in all genomes and show a high degree of length polymorphism. STR analysis can therefore be used for paternity assessment and for the analysis of criminal cases. These genetic marker are often used in population and conservation genetics but have not been applied to LWFG so far.

We have used ISSR-PCR, that amplifies DNA between STR elements, to characterise LWFG and related goose species. 15 polymorphic, i.e. variable DNA elements were found in LWFG which characterise this species. The DNA profiles of other goose species are different, so that potential hybrids become detectable. Most of the polymorphic DNA bands, seen in German birds, are also present in the wild LWFG from Russia. According to our analysis, there are less than 20% hybrids (paternal and maternal) in the German LWFG breeding flock.

The STR system is presently being set up; results are not yet available.

Conclusion: The present methods allow the selection of pure breeding stock of LWFG, that do not contain apparent hybrids with other goose species. The breeding stocks or released birds that derive from them can thus be improved by deleting about 20% of birds with dubious DNA profiles.

Genetic substructures of LWFG populations

Previous research by M. Ruokonen and colleagues (2000a,b, 2004) have demonstrated that the mtDNA of LWFG fall into 2 complexes: one haplotype complex is more abundant in Western (W1: 9 subtypes), the other more abundant in Eastern populations (E1: 6 subtypes). However, each geographic breeding population is composed of a mixture of these basic haplotypes. The haplotype abundances within a population is not fixed but fluctuates with time and through immigration or emigration. It is certain that birds with different haplotypes that are present in a population mix under natural conditions.

According to our sequence analyses of mtDNA, also the German and Scandinavian breeding stocks consists of birds that originate from both main lineages W1 and H1. Similar to the situation in natural population, parent birds of both types have mixed, thus increasing the ge-

netic variability within LWFG. This intermixing is evident from ISSR data (but also seen in wild birds from Russia).

From a point of view of breeding, such a mixing is even advantageous in that close inbred lines (increased homozygosity) are prevented. Since original Fennoscandinavian LWFG are not available for breeding programs, the release of apparently pure LWFG can be recommended from a practical genetic point of view, even if the actual haplotype composition is not identical to that of the original Scandinavian birds.

Work in progress:

Analysis of 28 LWFG of the German breeding stock in comparison to captive birds from Sweden and Finland is been carried out at Heidelberg. As reference material we had 7 LWFG samples from Russia that came from native breeding grounds. Samples from other goose species (*Anser albifrons, fabalis, anser, Branta leucopsis, B. bernicla, B. canadensis*) are also available for comparisons The genetic analysis has not been completed at present but provides sufficient results to make safe recommendations:

It is intended that all LWFG that are kept for breeding in Germany and Scandinavia will be genetically screened using:

- Nucleotide sequences of mtDNA (purity of maternal lineage)
- Genomic fingerprinting with ISSR-PCR (detection of hybrids between species)
- Microsatellite analysis (assessment of allele frequencies in different breeding stocks to avoid inbreeding and to detect foreign alleles)

Aims & recommendation:

The Heidelberg laboratory will make a recommendations about which individual birds should be included in breeding programs and which should be eliminated.

Only LWFG that have been genetically screened and approved will be included in the breeding and reintroduction programs.

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Appendix: Explanation of methods

PCR methods

The analysis of genetic differences within a species demands methods that have a high degree of resolution. Sequences of mtDNA are powerful tools to study phylogeny and molecular systematics; thy are sometimes uninformative at an intraspecific level. Furthermore, since mtDNA is inherited maternally, hybridisation and introgression can mask an unambiguous allocation of individuals to lineages and populations.

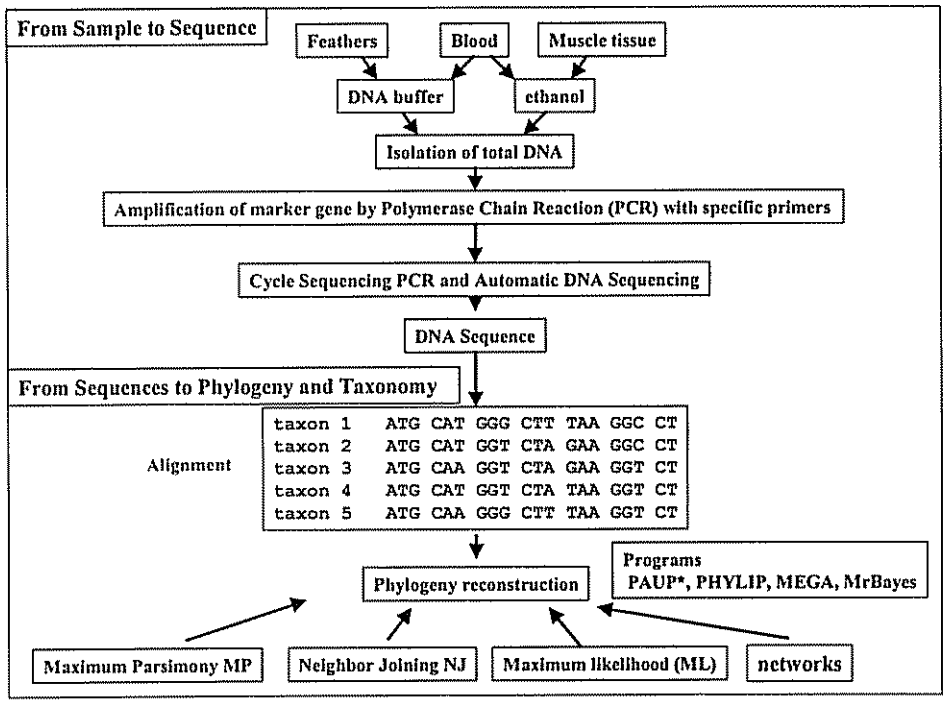


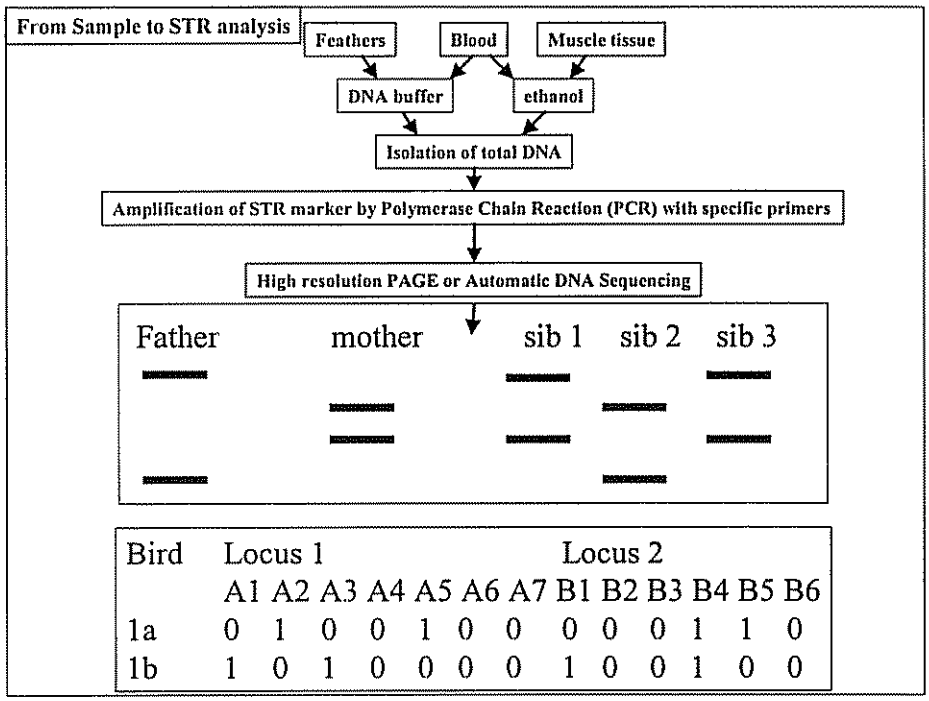
Fig. 1. From sample to DNA sequence and phylogeny

To overcome these problems, molecular markers of ncDNA, that are inherited by both sexes and that have a higher degree of resolution are more appropriate. These methods involve the amplification of polymorphic DNA markers by PCR and their separation by high resolution gel electrophoresis (often on agarose, better on polyacrylamide gels) or by capillary electrophoresis (using a DNA sequencer) (Hoelzel 1992; Karp et al. 1998; Storch et al. 2001; Frankham et al. 2002; Beebe and Rowe 2004).

Microsatellite (STR) analysis

As mentioned above, a vertebrate genome may contain over 20000 microsatellite loci, that are characterised by 10 to 20 fold repeats of short sequence elements, such as CA, TA, GACA

etc. The alleles of these loci show a high degree of length polymorphism. For each polymorphic STR locus several alleles exist that differ in the number of tandem repeats; they can thus be distinguished by size. An individual has two alleles for each locus: one derived from the father, the other from the mother. These alleles can be identical (homozygote) or not identical (heterozygote) (Hoelzel 1992; Karp et al. 1998; Storch et al. 2001; Frankham et al. 2002; Beebe and Rowe 2004). Since the sequences that flank these microsatellite loci vary between species, special efforts are needed to identify sequences that can be used to amplify the STR loci (Fig. 2). Several protocols have been published how to generate species-specific STR sequences (Lian et al. 2001).



Bird	Locus 1							Locus 2					
	A1	A2	A3	A4	A5	A6	A7	B1	B2	B3	B4	B5	B6
1a	0	1	0	0	1	0	0	0	0	0	1	1	0
1b	1	0	1	0	0	0	0	1	0	0	1	0	0

Fig. 2. Illustration of the inheritance of STR markers. Microsatellite PCR products are analysed by polyacrylamide gel electrophoresis (PAGE)
The lower box illustrates a 1/0 matrix that can be constructed from STR data. It can be analysed by phenetic methods that produce phenograms.

If information on PCR primers of microsatellites is not available, genomic fingerprint methods such as AFLP and ISSR provide an alternative (Hoelzel 1992; Karp et al. 1998; Storch et al. 2001; Frankham et al. 2002; Beebe and Rowe 2004). In my research group we have developed ISSR protocols for genetic characterisation of birds.

ISSR (*inter simple sequence repeats*) produces similar fingerprints as AFLP; it demands fewer experimental steps and is therefore easier to carry out. ISSR uses a single PCR primer, whose sequence is identical to common microsatellite motives, such as $(GACA)_4$. Since such loci are widely present in genomes and they occur in both orientations, a single primer is enough to amplify between 10 and 80 loci (i.e. DNA stretches between adjacent microsatellite loci) simultaneously. Since the PCR products differ in size they need to be analysed by high resolution PAGE or capillary electrophoresis (Fig. 3). The ISSR loci are inherited dominantly and since some of them are polymorphic they provide information of the genomic makeup of an individual. In practice, several of such ISSR primers are used, so that several hundred loci are available for analysis. The advantage of ISSR is, that the primers work universally in most animal and plant species. There is no need, to define PCR primers for an individual species, such as in microsatellite analysis. The results are plotted in a 1/0 matrix and evaluated by cluster analysis (such as UPGMA) that places individuals together based on the similarity of their ISSR band patterns.

ISSR can reveal population specific DNA bands, which can be useful to trace back individual bird to populations (Wink et al. 2002). Since ISSR loci are inherited by both sexes, this method also allows the analysis of hybrids and of sex (Wink et al. 1998; 2000). ISSR markers can also be used to infer phylogenies of closely related taxa, such as genera (Wink et al. 2002; Treutlein et al. 2003a,b).

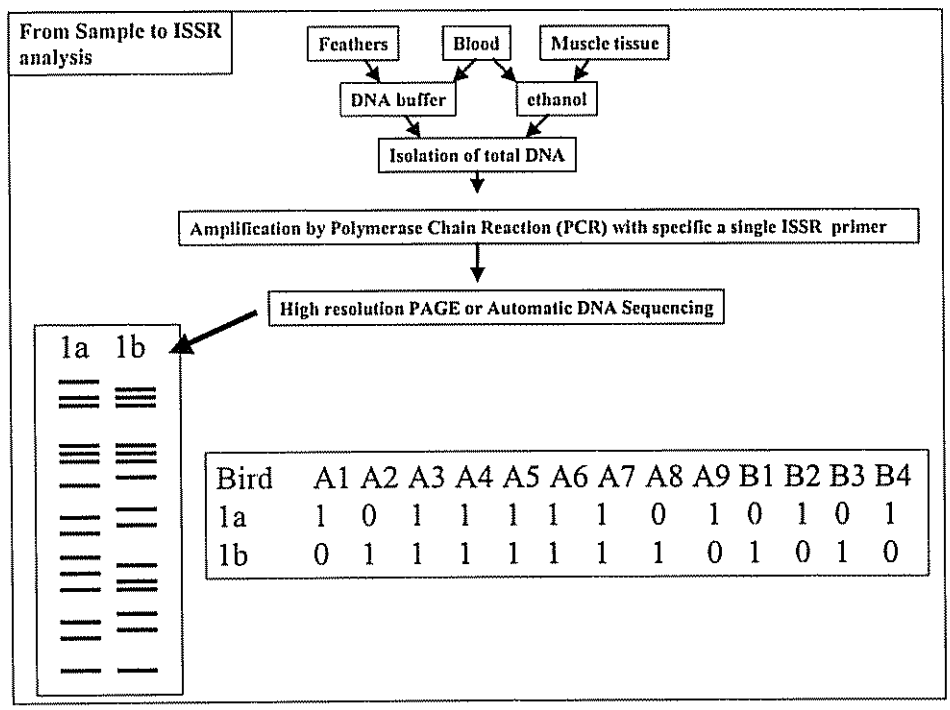


Fig. 3. Schematic outline of the ISSR method

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