Szent István University

THE ADAPTATION AND ISOLATION OF MICROSATELLITES FOR TURKEY AND GOOSE POPULATION STUDIES

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1. BACKGROUND AND OBJECTIVES

1.1. Background

To avoid world food shortage, production should be doubled in the near future (Baile 2000, Eggen 2012). Simultaneously, attention must be paid to sustain the biodiversity and adaptability and to preserve gene-pools of agricultural plant and animal species, which would secure the long-term survival of species and their populations. Methods to reach these goals include biotechnology and molecular genetics, which were among the most rapidly developing scientific areas in the last decades. For example, microsatellite markers were considered as modern and effective molecular tools when I started my work while in recent years genome sequencing of species is emerging rapidly.

The development of technology has increased the effectiveness of new generation sequencing. By now the entire genome of more than 80 vertebrates are known and this figure is increasing day by day (Ensembl Genome Browser - www.ensembl.org). However, the significance of molecular markers has not diminished yet since their application is cheaper and the assessment of results is much simpler and faster than those of genome sequencing. One of the most important directions of molecular studies is to learn the genetic background of agronomically important traits at the DNA level, e.g. identifying and tracking QTLs (Quantitative Trait Loci) by genetic markers. Molecular markers tightly linked to a QTL can be applied in MAS (Marker Assisted Selection) (Soller and Beckmann 1982, 1983).

Gene reserves are important because they might provide genetic resources for modern animal husbandry where breeding often reduces genetic variability of species. Poultry, especially turkey, provide very delicious and low-fat meat, which is getting increasingly popular. Additional advantages of keeping poultry compared to other species, such as cattle, include shorter generation time, simpler and cheaper farming (in
the case of intensive keeping) and the production of large amount of meat by hybrid varieties within a short period of time.

1.2 Objectives
- Adapting existing chicken microsatellite markers to turkey and goose. Selecting fluorescent primers for faster and more precise genotyping.
- Studying and characterising bronze turkey and curly-feather goose gene-reserve populations by adapted microsatellite markers.
- Isolating new microsatellite sequences from repetitive genomic DNA libraries for turkey and goose; testing and optimizing them as potential markers.

2. MATERIALS AND METHODS

2.1. Animal species and blood sampling
For population genetic studies, bronze turkey and curly-feather goose varieties kept at the model farm of the Department of Animal Breeding of the Agricultural and Management Sciences of Debrecen University (DE AGTC) were used. Blood samples of ten bronze turkeys were obtained from several turkey-keepers around Hungary and used for constructing the enriched microsatellite library.

Blood samples taken from 132 gene reserve curly-feather geese were used for population studies, while for microsatellite isolation and characterisation blood samples were obtained from four Hungarian geese and six Gourmaud hybrids.

Blood samples were collected from birds with individual wing tag into Falcon tubes containing 0.056 M trinatrium-citrate/0.086 M NaCl solution as anticoagulant.
2.2. Isolation, quality control and storage of DNA

2.2.1 DNA isolation by salt precipitation

DNA preparation was carried out by a modified version of the salt precipitation method developed by Miller and colleagues (Miller et al. 1988) using a solution containing an appropriate mixture of KCl, NaCl, EDTA and SDS, and centrifugation steps followed by isopropanol precipitation of the DNA. Precipitated DNA was washed with ethanol, dried and dissolved in TE puffer (0.01 M Tris-HCl 0.001 M EDTA).

2.2.2. DNA isolation by phenol-chloroform method

Blood samples were treated overnight at 55 °C in SET puffer (10 mM Tris-HCl, 50 mM EDTA, 200 mM NaCl, 0.5% SDS, pH. 7.8) containing 1 µg/ml Proteinase K enzyme (Fermentas) with continuous shaking. Phenol-chloroform extraction was used to remove the contaminating proteins (Blin and Stafford, 1976). DNA was precipitated using -20 °C ethanol, followed by centrifugation and washing using 70% ethanol at room temperature. After drying the DNA precipitate, it was dissolved in TE puffer.

2.2.3. Quality control and storage of DNA samples

The purity and quantity of DNA samples were determined by measuring their absorbance at 280 and 260 nms using a spectrophotometer. Samples with a minimum concentration of 0.05 µg/µl and 1.8-2 260/280 absorbance ratio were considered good quality and used used) in PCR reactions. All DNA samples were stored at +5 °C.

2.3. Detection of microsatellites

The size of the microsatellite alleles generated by PCR using fluorescently labelled primers was determined using an ABI PRISM 310 Genetic Analyser capillary gel electrophoresis instrument. In this method, the fluorescent label (6-FAM, TET, HEX, TAMRA) of the primers is excited by a laser and the emitted light of different wavelengths is measured and analysed by computing. It could be implemented that multiple fragments labelled by different fluorophores could be detected if they are different in size.
2.4. Data analysis

Data were analysed using the Populations (Langella 2011) and the Microsatellite Toolkit (Park 2001) software. The significance level of the differences between heterozygosity values was determined by the Markov chain method (Guo and Thompson, 1992) using the ARLEQUIN 3.5 software (Excoffier et al. 2005).

Expected heterozygosity ($H_e$) and observed heterozygosity ($H_o$), the indicators of for population heterogeneity were calculated. The fixation index, reflecting the difference of a population from the Hardy-Weinberg equilibrium, was calculated on the basis of the ratio of the two indicators I also examined the number of alleles detected in the population, their frequency ($p_i$), the polymorph information content (PIC) and the distance between species ($D_A$).

2.5. Constructing microsatellite-enriched DNA libraries

Libraries enriched in microsatellite sequences were prepared following the method of Glenn and Schable (2005). DNA from three turkey individuals (20 µg of each) was pooled to construct two libraries. From the pooled DNA, 30 µg was digested by Rsal and HaeIII restriction enzymes. 500 to 1200 bp long fraction from the digested DNA was isolated and an adapter sequence was ligated to the end of the fragments in order to assist the next enrichment step. To prepare the double-strand adapter (Figure 1), two complementary oligonucleotides were mixed in equimolar amount, heated to 95 °C in a PTC-200 (MJ Research) PCR machine, and then slow cooled down to room temperature. Ligation of the adapter to the digested DNA was tested by PCR using adapter-specific primers.

\[
5' \text{AGGTACCAGCCATATGGGCAGCATGC } 3'
\]
\[
3' \text{TTAGGTACCAGCCATATGGGCAGCATGC } 5'
\]

Figure 1. Structure of the double-strand adapter after annealing

Enrichment was performed using biotin-labelled oligonucleotides and streptavidin-coated magnetic beads (MagneSphere, Promega). Sequences containing putative microsatellites were amplified by PCR and
cloned into pGEM T-Easy vector. The sequence of the inserts was determined by sequencing.

The size of the inserts in pGEM-T Easy plasmid was determined by colony PCR using M13 forward and reverse primers. PIMA-PCR was used to select inserts with sequence repeats. In addition to the M13 forward and M13 reverse primers, a CA primer with the sequence of (AC)$_8$DN was used.

**Figure 2. Steps of establishing an enriched library.**

Adapter sequences were ligated to fragments obtained by restriction enzyme digestion and selection of 500-1200 bp size fragments. Using streptavidin beads biotin-labelled repetitive sequences were separated and using PCR they were amplified into double-strand fragments. Fragments were ligated into pGEM T-Easy vector and their sequence was determined.

The sequence of inserts was determined with a ABI PRISM 310 Genetic Analyser (Applied Biosystems) using the Big Dye Terminator 3.1 kit. Specific primers were designed by the Oligo Explorer software for inserts in which the sequence bordering the repeat was long enough to design 18 to 25 base long primers.
For optimisation the designed microsatellite primers, I determined their optimal annealing temperature using the Oligo Analyser software, set the MgCl₂ concentration, and also established the thermal profile of the PCR reactions.

3. RESULTS

3.1. Adapting microsatellites

3.1.1. Bronze turkey

Based on literature data, 56 microsatellite markers were selected for the turkey studies. Specific PCR product was detected for 46 (82.1%) microsatellites, of which 20 (35.7%) were polymorph. The combination of primers, MgCl₂ concentration used in the PCR reaction, PCR temperature profile, the length of the products and the fluorescent label made creating of detection groups possible using the ABI PRISM 310 Genetic Analyser.

To analyse the turkey population, the two groups as the subjects of the analysis contained 15 microsatellites (group I and II) with 8 (Table 1) and 7 (Table 2) microsatellite sets in group 1 and 2, respectively. Multiplex groups (3) were created to multiply the product by considering size and fluorescent mark.

Table 1: Microsatellite group I. The table contains the name of microsatellite primers, their fluorescent mark and the size of the final products.

<table>
<thead>
<tr>
<th>Microsatellite name</th>
<th>Fluorescent mark</th>
<th>Product size (bp)</th>
<th>Multiplex PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADL292</td>
<td>FAM</td>
<td>118-132</td>
<td>A</td>
</tr>
<tr>
<td>MCW18</td>
<td>FAM</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>ADL293</td>
<td>TET</td>
<td>81-108</td>
<td>B</td>
</tr>
<tr>
<td>ADL272</td>
<td>TET</td>
<td>160-168</td>
<td></td>
</tr>
<tr>
<td>ADL149</td>
<td>TET</td>
<td>222-228</td>
<td></td>
</tr>
<tr>
<td>ADL266</td>
<td>HEX</td>
<td>81-104</td>
<td>C</td>
</tr>
<tr>
<td>ADL150</td>
<td>HEX</td>
<td>121-138</td>
<td></td>
</tr>
<tr>
<td>MCW80</td>
<td>HEX</td>
<td>276-278</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: **Microsatellite group II.** The table contains the name of microsatellite primers, their fluorescent mark and the size of the final products.

<table>
<thead>
<tr>
<th>Microsatellite name</th>
<th>Fluorescent mark</th>
<th>Product size (bp)</th>
<th>Multiplex PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCW83</td>
<td>HEX</td>
<td>65-75</td>
<td>D</td>
</tr>
<tr>
<td>ADL146</td>
<td>FAM</td>
<td>193-197</td>
<td></td>
</tr>
<tr>
<td>ADL142</td>
<td>TET</td>
<td>99-111</td>
<td>E</td>
</tr>
<tr>
<td>ADL361</td>
<td>HEX</td>
<td>106-120</td>
<td></td>
</tr>
<tr>
<td>ADL353</td>
<td>FAM</td>
<td>142-156</td>
<td></td>
</tr>
<tr>
<td>LEI143</td>
<td>TET</td>
<td>210-218</td>
<td>F</td>
</tr>
<tr>
<td>ADL191</td>
<td>TET</td>
<td>134-140</td>
<td>G</td>
</tr>
</tbody>
</table>

3.1.2. **Curly-feather goose**

Based on literature data, 26 microsatellites were selected to adapt them for goose. Specific product was obtained with 16 markers (61%) in geese. Nine microsatellites (34.6%) were polymorphic, of which five were selected for further studies and were grouped (Table 3) according to their fluorescent label.

Table 3: **Microsatellite group to examine curly-feather goose.** The table contains the name of microsatellite primers, their fluorescent mark and the size of the final products.

<table>
<thead>
<tr>
<th>Microsatellite name</th>
<th>Fluorescent mark</th>
<th>Product size (bp)</th>
<th>Multiplex PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADL0142</td>
<td>TET</td>
<td>196 - 204</td>
<td>A</td>
</tr>
<tr>
<td>MCW0018</td>
<td>FAM</td>
<td>206 - 208</td>
<td></td>
</tr>
<tr>
<td>ADL0361</td>
<td>HEX</td>
<td>103 - 115</td>
<td>B</td>
</tr>
<tr>
<td>LEI0120</td>
<td>FAM</td>
<td>270</td>
<td>C</td>
</tr>
<tr>
<td>MCW0080</td>
<td>HEX</td>
<td>206 - 238</td>
<td></td>
</tr>
</tbody>
</table>

3.2. **Population genetic studies**

Based on the allele composition (allele number and fragment size) of the examined bronze turkey and curly feathered goose individuals, calculations were made to characterise both populations. Observed and expected heterozygosity was calculated for the typical main and rare alleles. Fixation index and the PIC value, characteristic for heterozygosity, were also calculated and a dendrogram was also constructed to have a clearer and simpler picture about the genetic structure of the populations.
3.3. Isolating new microsatellites

3.3.1. Turkey

The relatively few number of adapted microsatellite markers, which I could use in my studies prompted me to develop additional markers. I, therefore, constructed a turkey microsatellite library, from which 167 repeat sequences were selected and 61 primer pairs were designed.

3.3.2. Goose

A similar library was also prepared for goose, from which 32 clones were selected and 5 primers were designed.

3.3.3. Mapping turkey microsatellites to the turkey genome

Publishing the genome sequence of turkey made it possible to compare the isolated microsatellite sequence data with the turkey sequence database.

4. CONCLUSIONS AND RECOMMENDATIONS

Adapting microsatellites is important to examine species that do not have appropriate number of genetic markers (Andres and Kapkowska 2011, Quing-Ping et al. 2009). This was the situation with turkey (Meleagris gallopavo) and goose (Anser anser) when I started my research. Chicken (Gallus gallus) has the most detailed genetic map and the largest number of genetic markers among poultry (Groenen et al. 2000, Hillier et al. 2004, Takashi et al. 2004, Cogburn et al. 2007) so it was quite obvious to use chicken microsatellites to study other poultry species.

In my experiments chicken microsatellites were successfully adapted for both turkey and goose. For turkey, more than 80% of the tested chicken markers produced a fragment, while in goose more than 60% of the markers gave positive result. These figures are in good agreement with literature data showing that the ratio of adaptable microsatellites between two species decreases as their taxonomic distance
increases (Andres and Kapkowska 2011, Quing-Ping et al. 2009). It is interesting, however, that approximately 35% of the tested chicken markers were polymorph in both turkey and goose populations, i.e. those markers were suitable for population genetic studies.

4.1. Increasing the efficiency of population genetic analysis

Altogether, three groups were successfully formed from the adapted microsatellites. In the two turkey groups, there are 15 microsatellites altogether which can be amplified by five multiplex and two single PCR reactions. In one of these two groups, the separation of maximum 16, 81 to 278 bp long, DNA fragments is possible using three different fluorescent labels, if all the microsatellites are heterozygotes. In the second turkey microsatellite marker group maximum 14, 65 and 218 bp long, alleles can be separated. The third group is suitable to study goose and contains only 5 microsatellite markers with 103 bp to 238 bp length.

My studies revealed that these microsatellite groups were suitable for population analyses although the development of additional markers are necessary because some microsatellites for both turkey and goose in were proved to be monomorphic in the examined populations. Their possible change is recommended if they have one or two alleles in other populations, as well. However, I could apply these monomorphic microsatellites as internal markers during capillary electrophoresis because their size is constant and thus the proper operation of the machine can be controlled by them.

The development of additional markers is feasible since, in theory, the number of microsatellites in multiplex PCR can be increased up to 10 to 15 (Ajzenberg et al. 2010).

4.2. The genetic examination of indigenous poultry populations

The number and frequency of alleles were examined. The number of alleles for all 15 microsatellites was low (1 to 4), which is lower than the typical value for turkey (Reed et al. 2003a, Reed et al. 2003b, Latch et al. 2002). The low number of alleles can indicate inbreeding, which can result in decreased fitness of the population (Pecsenye 2006). I observed for each marker, but one (LEI43) that one allele is more frequent than the others and is present in more than 60% of the detected haplotypes. In wild turkey, due
to the low number of examined patterns, much fewer alleles were found although the 111 bp allele of the LEI43 marker was only present in wild turkey.

The expected \((He)\) and observed \((Ho)\) heterozygosity of populations were determined from genotype data and I found that there was only a minor difference between the two values in most cases Statistically significant difference \((P<0.05)\) was found between the H-values of only two microsatellites, ADL150 and ADL293. The overall difference for the population \((He: 0.28; Ho: 0.312)\) was not significant, either.

Heterozygosity values in wild turkey showed a similar picture and no significant difference was found in any marker.

The fixation index, which characterises the deviation from Hardy-Weinberg equilibrium, was calculated from the \(H_e\) and \(H_o\) values. In the case of bronze turkey one of the microsatellites, ADL150, was homozygote. The positive value of ADL293, ADL272, ADL146, LEI43 and ADL191 refers to the lack of heterozygotes. The other microsatellites (ADL293, ADL266, MCW80, ADL353, ADL142, MCW83, ADL361) had a negative value and excess heterozygotes could be detected in the population. In wild turkey, the calculation of \(F_{IS}\) value is not applicable for four microsatellites (ADL292, MCW18, ADL146, ADL191), because they had homozygote genotype. ADL272 and ADL149 yielded in positive values, which refers to the lack of heterozygotes. In the case of the other microsatellites the fixation index had a negative value, which also refers to the excess of heterozygotes.

The PIC value was smaller than 0.25 for the ADL146, ADL150, ADL292, ADL293, MCW0080 markers in bronze turkey, thus these microsatellites are slightly or hardly informative. Seven microsatellites (ADL142, ADL149, ADL191, ADL266, ADL353, ADL361 and MCW83) belonged to the category of moderately informative microsatellites. Their PIC value varied between 0.25 and 0.5. Only one microsatellite, LEI43, was significantly informative as its PIC value was greater than 0.5. For MCW18, the PIC value was zero indicating that this is a monomorphic microsatellite with one allele, i.e. homozygous to the marker.

PIC values were also calculated for wild turkey and for four microsatellites, ADL146, ADL191, ADL292, MCW18, the result was
zero indicating that they were homozygous. With the exception of ADL142, which was greatly informative, all other markers turned to be moderately informative (0.5 > PIC > 0.25).

A dendrogram was used to show the genetic similarities, differences and the relationship between individuals.

Several large groups, with small genetic distance, can be observed on the dendrogram. Keeping the birds in cages might be the explanation for this, although males are rotated between the cages annually in order to avoid inbreeding depression. Wild turkey individuals are scattered throughout the dendrogram, i.e. they do not form a separate group or sub-population. Although there is no clear explanation for this in this moment, it is possible that the examined wild turkey individuals had introgression from bronze turkey, in particular that the origin of that wild turkey individuals is not clear.

4.3. The genetic analysis of curly-feather goose population

I used five microsatellites to study this population. Although more markers would be necessary for a satisfactory analysis of the genetic background of the population, a preliminary survey, based on my data, is possible. The number of alleles in the curly-feather goose population was also low (no more than three per marker). Similarly to turkey, certain alleles were in larger proportion in some individuals of the population. The expected \((H_e)\) and observed heterozygosity \((H_o)\) values were also calculated.

In the experiments, three microsatellites, ADL142, ADL361 and MCW18, showed significantly lower values of heterozygosity than expected. One of the microsatellites, LEI120, was homozygous, while one microsatellite, MCW80, had significantly higher heterozygosity value. The total observed heterozygosity value of the population (0.0191) was significantly lower than the expected value (0.3169).

In the case of homozygote LEI120 microsatellite, the PIC-value was zero as expected. The PIC-value of one microsatellite, ADL361, was hardly informative (it was smaller than 0.25). Three other markers, ADL142, MCW18 and MCW80, were moderately informative since their PIC-value was between 0.25 and 0.5.

To examine the genetic similarities in the goose population, a dendrogram was prepared. It could be observed that individuals with the
same or similar genotype are very common, which indicates that inbreeding occurred within the population and/or under the free-range keeping of the population certain individuals might have produced more offsprings, which were included into the studies.

4.4. New turkey and goose microsatellites

DNA libraries enriched in repetitive (dinucleotide) sequences were created from both species in order to isolate new microsatellite markers. Sixty-one new microsatellite markers for turkey and five for goose were developed. The sequence of the markers was determined and PCR primers were designed for amplification. The primers were tested in PCR and amplification conditions were optimised for them. These markers seem to be suitable to study genetic variability of populations and to explore the genetic background of economically important, valuable traits.

We attempted to map the newly isolated turkey microsatellites to a turkey genetic map by an international collaboration. The position of eleven markers was determined by our partners, Kent M. Reed and his team, on the consensus genetic map of turkey using a Minnesota mapping population and was also mapped to the comparative genetic map of chicken (Reed et al. 2005). For the mapping, I have performed a preliminary genotyping of certain individuals of the mapping population using the microsatellite markers in order to see whether they are polymorphic and thus suitable for genetic mapping.

The turkey genome was sequenced and became available in 2010, which made it possible to determine the genomic position of the newly isolated microsatellites. Six of the 61 new microsatellite marker did not have any homology in the database, thus they could not be assigned to chromosomes. The position of the rest of the markers, including those that were mapped by the collaborators, could be annotated to the genome. In the case of three markers, MGP012, MGP031 and MGP43, however, the result of the genome annotation and the genetic mapping was different. I have annotated MGP012 to chromosome 13 and MGP031 and MGP43 to chromosome 15, while genetic mapping put MGP012, MGP31 and MGP043 to chromosome 11, 2 and 19, respectively. This discrepancy can be explained by the not properly assembled genome sequence, which is
also continuously being edited and updated. In particular, repeat sequences are difficult to assemble, thus further studies are needed to precisely position such markers on the genome.
5. New scientific results

1. Forty six chicken microsatellites were successfully adapted for turkey studies. Depending on the size of microsatellite alleles, two groups were formed to examine adapted microsatellites more efficiently and cost effectively. In one of the groups eight microsatellites could be multiplexed.

2. Fifteen adapted microsatellite markers were used to study the genetic variability of the bronze turkey population from Debrecen and some wild turkey individuals. The obtained results were assessed and provided to the breeder for assistance in further breeding tasks.

3. After constructing a turkey microsatellite library, 167 DNA sequences containing dinucleotide repeats were isolated of which 109 were suitable for primer design and 68 had unique sequence. Blast search of these 68 sequences revealed that 61 newly isolated sequences are not present in gene banks and specific primer pairs were designed for them.

4. Sixteen microsatellite primer pairs were successfully adapted for goose in order to study their different populations. Five of the microsatellites were placed into one multiplexing group.

5. Groups of five microsatellites were used to survey the genetic variability of the curly-feather goose population of DE AGTC. The obtained results were assessed and handed over to the breeder to assist in further breeding tasks.

6. A library enriched in goose microsatellites was successfully constructed and by sequencing 32 clones 11 unique microsatellite sequences were identified of which 5 new markers were developed by designing primers.
6. LIST OF PUBLICATIONS

6.1 Publications on the subject of the dissertation

Papers in scientific journals


Papers at conferences and workshops (proceedings)


Lectures at conferences (abstract)


6.2 Other publications

*Papers in referred scientific journals*


Lectures at conferences (abstract)


