PROTOCOL 5.

Mitochondrial DNA analysis of DNAs isolated from Balkan chamois bone remains

Things to be done before the start:

- **Dilute DNA samples down to the concentration of 1ng/µl.**
- **Dilute SSR primers down to final concentration of 10 µM.**
- **Gradually thaw 10 x PCR Buffer and dNTPs at temperature of 4 – 8 °C.**
- **Prepare 5X TBE Electrophoresis Buffer (54 g Tris base, 27,5 g of boric acid and 20 ml of 0,5 M EDTA (pH 8.0) in 1000 ml distilled water.**
- **Prepare 6X Gel-loading Buffer (0,25 % bromphenol blue, 0,25 % xylene cyanol, 40 % (w/v) sucrose in distilled water).**

STEPS INVOLVED IN MITOCHONDRIAL DNA ANALYSIS:

PART 1. PCR AMPLIFICATION OF MITOCHONDRIAL PARTIAL CYTOCHROME B GENE AND ITS CONTROL REGION
PART 2. AGAROSE-GEL ELECTROPHORESIS OF PCR AMPLIFIED MITOCHONDRIAL REGION
PART 3. AUTOMATED DNA SEQUENCING OF PCR AMPLIFIED MITOCHONDRIAL REGION AND GEN BANK COMPARISON OF PCR AMPLIFIED REGION

PART 1. PCR AMPLIFICATION OF MITOCHONDRIAL PARTIAL CYTOCHROME B GENE AND ITS CONTROL REGION

1) DNA samples

- DNA samples were isolated from: 1) Balkan chamois molar remains (Velebit (Cro) 1893), 2) Balkan chamois maxilla remains (location and date unknown).

2) PCR amplification

- PCR mix (fluorescently lebelled SSR primers developed by (Radosavljević et al. 2010, 2011):

74,40 µl H2O
16,00 µl 10 x PCR Buffer (TaKaRa®)
12,80 µl dNTP (TaKaRa®)
8,00 µl mt primer L14724 (5'- CGAAGCTTGATATGAAAAACCATCGTTG -3') 10 µM
8,00 µl mt primer H15149 (5'- AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA -3') 10 µM
0,80 µl TaqHS polimerase (TaKaRa®)
120,00 µl divide in eight 0.2 ml PCR tubes (15 µl of PCR mix in each tube) and add 5 µl of each DNA (c = 0,5, 1, 5 and 10 ng/µl) to get final volume of 20 µl

- PCR profil

94 °C 4 min;
94 °C 30 sec, 50 °C 30 sec, 72 °C 45 sec (25 cycles);
72 °C 10 min;
12 °C (forever)

PART 2. AGAROSE-GEL ELECTROPHORESIS OF PCR AMPLIFIED MITOCHONDRIAL REGION

1) Seal the edges of plastic tray to form a mold and set it on a horizontal section of the bench.
2) In an Erlenmeyer flask prepare 120 ml 0,5X TBE buffer and add 1,5 g agarose
3) Heat in a microwave oven until the agarose dissolves.
4) Cool the melted agarose solution to 60 °C and pour into the mold.
5) Set the comb and allow gel to set completely (45 minutes at room temperature).
6) Remove the comb carefully
7) Mount the gel in the electrophoresis tank and cover with 0,5X TBE buffer to a depth of ~ 2 mm.
8) Mix 5 µl of PCR product with 1 µl of 6X Gel-loading Buffer and load slowly this mixture into the slot of submerged gel. Load all samples and DNA size standard.
9) Close the lid of gel tank and attach the electrical leads so that the DNAs migrate toward the positiv anode.
10) Turn on Power supply (~ 120 V).
11) After 30 minutes turn off the electric current and immerse the gel in 0,5X TBE buffer containing gel-red dye (0,5 µg/ml) for 30 minutes.
12) Set the gel to UV-transiluminator of DigiGenius® Gel Documentation System and make photo using camera with orange filter and protective shield.

* Wear nitril gloves at all times when handling liquids or gel!

PART 3. AUTOMATED DNA SEQUENCING OF PCR AMPLIFIED MITOCHONDRIAL REGION AND GEN BANK COMPARISON OF PCR AMPLIFIED REGION

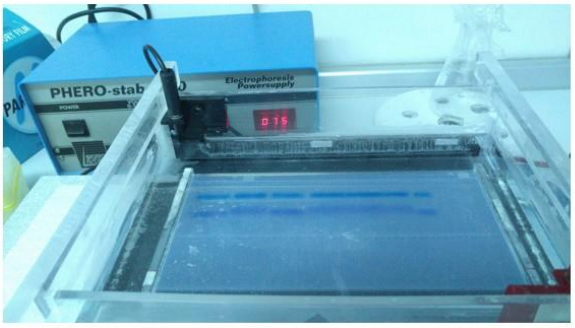
1) To clean-up PCR product from unincorporated primers and degrades unincorporated nucleotides prepare the following reaction mixture per each sample:
   5 µl PCR mixture (directly after completion of PCR)
   0.5 µl Exonuclease I (Exo I) (10 u)
   1 µl FastAP™ Thermosensitive Alkaline Phosphatase (1 u)
2) Mix well and incubate at 37°C for 15 min.
3) Stop the reaction by heating the mixture at 85°C for 15 min.
4) Mix 5ul of purify PCR Product and 5ul of 5uM single PCR primer.
5) Close 0.2 ml PCR tubes and pack in a protective cardboard box.
6) Make an order on Macrogen web-site (dna.macrogen.com/eng → Order → EZ Seq).
7) Send samples to Macrogen Inc by DHL or Fedex.
8) The sequence results shall be delivered to the email address as .ab1 files
9) Launch Genious® software and import .ab1 files
10) Click two .ab1 files that represent different (complementary) strands of the same DNA sequence and assemble them in a consensus sequence.
11) Save consensus sequence as a .txt file
12) Open BLASTN (https://blast.ncbi.nlm.nih.gov) and paste consensus sequence.txt file.
13) Click BLAST and search nucleotide databases using a nucleotide query.

References:

1) https://blast.ncbi.nlm.nih.gov
2) Rodríguez et al 2009. Cytochrome b Phylogeography of Chamois (Rupicapra spp.). Population Contractions, Expansions and Hybridizations Governed the Diversification of the Genus. Journal of Heredity 100(1):47–55.
3) Sambrook and Russell 2001. Detection of DNA in agarose gel. In: Molecular Cloning – A Laboratory Manual, Third Edition, 5.14-5.17. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Laboratory equipment and accessories used for mitochondrial DNA analysis

ProFlex® PCR System



Agarose-gel Electrophoresis

geneious

Geneious® Bioinformatics Software

DigiGenius ® Gel Documentation System

Micropipette Set