LABORATORY MANUAL FOR WILDLIFE MOLECULAR FORENSICS

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TABLE OF CONTENTS

GENERAL PROCEDURES

SAMPLE COLLECTION FOR DNA EXTRACTION

Fresh blood
1. pipette (or translocate otherwise) one to several drops of liquid blood onto paper towel
2. mark sample by circling stain with pen and indicating sample ID and other information
3. allow to dry
4. store in ziplock bag at -70?C (can also be stored at room temperature for extended periods)
   instead of paper towel q-tips can be used by dipping them into fresh blood and storing them as above

Blood stains, coagulated, and dry blood
store in ziplock bag or vial at -70?C

Feathers
1. pluck one to several feathers from subject (contour feathers work well)
2. cut feathers 1-2mm above feather base
3. store bases in vial at -70?C

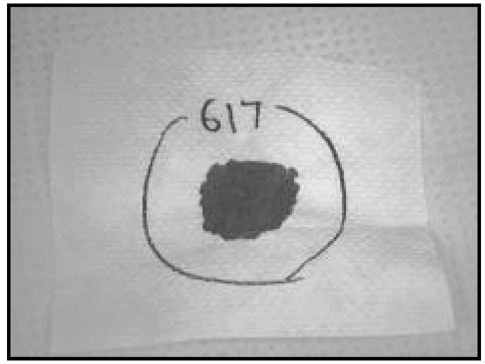


Figure 1: Paper towel with blood stain and sample ID.

Muscle and skin tissue
store in ziplock bag or vial at -70?C

Fin tissue collection in the field
1. cut out desired portion of fin
2. spray fin clip with 95% Ethanol while holding over tray with forceps
3. place fin clip into vial filled with 95% Ethanol
4. add label with sample ID, date, location etc.
5. repeat for each sample while cleaning scissors and forceps between samples with 95% Ethanol
   this is necessary to avoid both infection of subsequent fish and contamination of samples
6. store vials at room temperature

Plant tissue
store in ziplock bag with silica gel at room temperature or store at -70?C

DNA EXTRACTION

DNA EXTRACTION FROM ANIMAL TISSUE

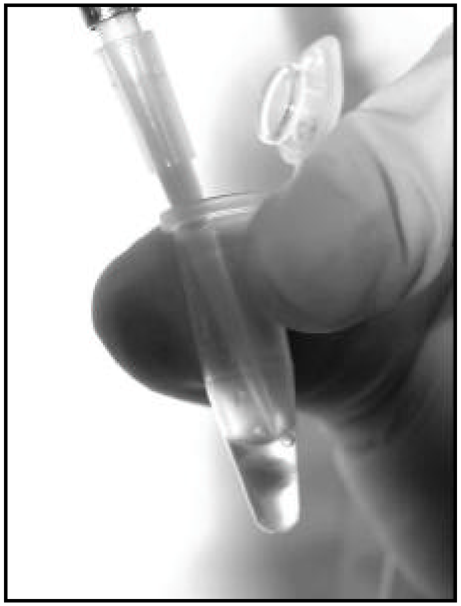


Figure 2: Preparing sample for DNA extraction.

1. label set of Ependorf tubes (1500?l)
2. move an aprox. 3mm x3mm large tissue sample into assigned tube
3. add 200?l extraction buffer
4. add 1?l Proteinase K (Figure 2)
   perhaps brake sample in tube with 100ul-pipette tip
5. incubate in waterbath at 55?C for 3 hrs
6. spin down (hold pulse button until centrifuge reaches aprox. 3000rpm)
7. add 100?l Phenol under fume hood
8. shake each tube
9. incubate at 55?C for 15min while shaking tubes in 3-4min intervals
10. spin down
11. add 100?l Chloroform under hood
12. shake each tube
13. centrifuge at 12000rpm for 5min
14. label new set of 1500?l tubes
15. move top layer (with pipette set to aprox. 400?l) to new tube (Figure 3)
16. add 200?l Chloroform
17. shake tubes
18. centrifuge at 12000rpm for 5min
19. label new set of tubes
20. move top layer to new tube
21. add 400?l 100% Ethanol
22. incubate at -20?C overnight
    if in a hurry samples can be incubated at -70?C for 3-4hrs
23. centrifuge at 12000rpm for 20min
24. decant Ethanol and shake out remaining liquid
25. add 300?l 70% Ethanol
26. centrifuge at 12000rpm for 10min
27. decant Ethanol and thoroughly shake out remaining liquid
28. dry at 20psi for 30 min
29. add 100?l TE
30. flick up DNA pellet
31. keep at room temperature for 30min
32. store at -20?C

Figure 3: Removing supernatant.

DNA EXTRACTION FROM BLOOD

1. label 1500?l Ependorf tubes
2. add 5-10?l whole blood or a 1cm x 1cm piece of soaked tissue into each tube
   blood samples should be frozen or fresh

3. add 1000?l nanopure water
4. incubate at room temperature for 30min-1hr
5. centrifuge at 12000rmp for 2min
6. remove supernatant with pipetter
   careful to not disturb pellet
7. add 200?l chelex solution (with 1000?l pipette tip)
   chelex solution should be stirred at moderate speed to keep beads in suspension
8. incubate in waterbath at 56?C for 30min
9. vortex at high speed for 10sec
10. incubate in boiling waterbath for 8 min
11. vortex at high speed for 10sec
12. centrifuge at 12000rpm for 2min
13. use 20?l of supernatant per 50?l PCR reaction (use 20?l ddH2O per tube instead of 40?l)
    vary used volume if amplification unsuccessful
14. store at -20?C
    vortex and centrifuge samples for 2min at 12000rpm before each use

<u>DNA EXTRACTION FROM PLANT TISSUE</u>

1. label Ependorf tubes (1500?l)
2. warm up 2xCTAB Extraction buffer in hot water while stirring
3. cut aprox. 2mm piece off tip of 1000ul pipette tip
4. move 500?l CTAB into each tube
5. add 1?l 2-Mercaptoethanol into each tube (under fume hood)
6. grind dry or frozen plant tissue (aprox. 1cm x 1cm size) in mortar
7. add into tube (submerge into CTAB)
8. incubate in water bath at 60?C for 1hr
9. spin down
10. add 500?l Chloroform (under hood)
11. shake tubes
12. let sit for 10min
13. centrifuge at 12000rpm for 5min
14. label new set of tubes
15. transfer (with pipetter set to aprox. 500?l) upper layer to new tube (under hood)
16. add 500?l Chloroform
17. shake tubes
18. let sit for 10min
19. centrifuge at 12000rpm for 5min
20. label new set of tubes
21. transfer upper layer to new tube
22. add 1000 ?l 100% Ethanol
    should end up with white flakes in yellow-ish liquid
23. incubate at -20?C overnight
24. centrifuge at 12000 rpm for 5 min
25. decant alcohol and shake out remaining liquid
    careful not to remove pellet
26. dry at 20psi for 20-30min
27. add 100?l TE
28. disturb pellet with pipette tip
29. add 10 ?l 3xSodium Acetate
30. add 250 ?l 100% Ethanol
31. vortex at high speed for 5sec
32. incubate at -20?C for 1hr
33. centrifuge at 12000rpm for 10min
34. decant ethanol and carefully shake out tubes
35. add 1000?l 70% ethanol
36. mix well with pipetter (1000?l tip) until dissolved
37. centrifuge at 12000rpm for 10min
38. decant alcohol and carefully shake out tubes
    repeat the last 4 steps if CTAB (white layer on bottom) is still visible in the tube
39. dry at 20psi for 30min to 1hr
40. store at -20?C

DNA PURIFICATION

1. move 50?l of PCR product onto Parafilm
2. insert filters into Microconcentrator tubes (red side up)
3. label Microconcentrator tubes
4. move pcr product from Parafilm into filter tube while leaving oil behind
   do not pipette sample directly onto filter
5. centrifuge at 2500rpm for 15min
6. cut lids off new Microconcentrator tubes
7. label tubes on side
8. invert filters into new tubes (white side up)
9. centrifuge at 3500rpm for 5min
10. label 1500?l Ependorf tubes on lid and on side
11. move resulting liquid (on Microconcentrator tube bottom) into Ependorf tubes with pipetter set to 7?l
12. wash out old tube with 7?l nanopure water and add to new tube
13. store at -20?C

Testing for successful purification:

1. prepare 1% agarose gel
2. put dye-dots on Parafilm
3. add 1?l purified DNA to each dot
4. add 3?l TBE buffer from electrophoresis apparatus
5. fill samples into gel grooves with pipetter set to 7?l
6. pipette 7?l 100bp ladder into first groove
7. pipette 2.5?l biomarker into last groove
8. run gel at 170V

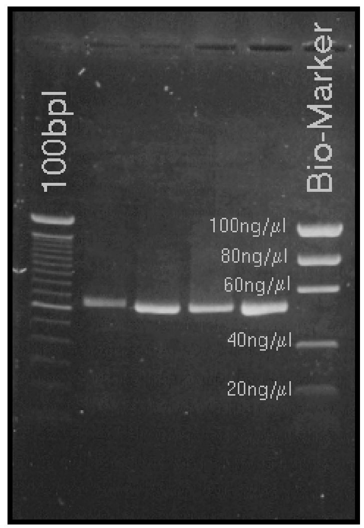


Figure 4: Gel with purified DNA and bio-marker.

DNA SEQUENCING

We have our samples sequenced by the University of Iowa Sequencing Facility, Ames, IO. Samples for sequencing are prepared as follows:

1. purify desired DNA samples as described in section “Purification” Steps 1-12
2. label 1500?l Ependorf tubes for primers (lid and side)
3. add 15?l nanopure water
4. add 5?l primer (from working solution)
5. seal primer and purified DNA tube lid with Parafilm (1cm x 3cm strip)
   one set of primers is sufficient for all samples sent in a single batch that were amplified with that primer pair
6. fill out sequencing order form
7. send in reinforced envelope
8. Sequences can be retrieved via FTP from biocomp.unl.edu

DNA AMPLIFICATION

Figure 5: Thermal Cycler with samples.

1. write out component quantities per tube
- ? 1?l primer 1
- ? 1?l primer 2
- ? 5?l buffer
- ? 4 ?l nucleotides (dNTP)
- ? 0.5?l taq polymerase
- ? 40?l nanopure water
2. multiply with number of tubes
3. label 500?l PCR tubes for each sample
4. add primers to master solution tube (1500?l Ependorf tube)
5. add buffer
6. add nucleotides
7. add taq polymerase

keep in freezer until needed and return immediately

8. add nanopure water

use calculated quantity plus 10% of calculated quantity of water

9. add 48?l of master solution in each PCR tube
10. add 1?l template
11. add one drop of oil
12. turn on thermal cycler and start
13. move PCR tubes into thermal cycler (Figure 5)

first allow thermal cycler to reach apprx. 85?C to prevent the activation of non-desired enzymes with lower working temperature

14. when program is done turn thermal cycler off
15. store PCR product at -20?C

Testing for successful amplification:

1. prepare agarose gel
2. put dye-dots on Parafilm
3. add 5?l PCR product per dye dot
4. load DNA-dye mixture into gel grooves (pipetter set to 7?l)
5. run gel
6. mark all PCR tubes containing amplified DNA (toss the rest)
7. mark all tubes containing original DNA that amplified

RESTRICTION ENZYME DIGEST

FINDING RESTRICTION ENZYMES AND SITES

1. -obtain sequence (.e.g. from biocomp)
2. -locate bases in sequence that are variable (on a desired level)
3. -paste sequence into WebCutter
4. -run search on restriction enzymes that recognize sites anywhere within the sequence

output is a list of restriction enzymes with the name, the recognicion site and the position of the cut(s) in the pasted sequence

5. -look for a restriction enzymes that cut at the positons that are variable
6. find all the recognition sites of a choosen restriction enzyme
7. check alligned sequences (exclude outgroup to avoid gaps, otherwise the bp numbers are not conform) to make sure the sites are real
8. calculate fragment sizes

RESTRICTION ENZYME DIGEST

1. write out component quantities per tube:
   reactions without BSA:
   ? 0.5?l restriction enzyme
   ? 1?l buffer
   ? 6?l nanopure water
   reactions with BSA:
   ? 0.5?l restrictions enzyme
   ? 1?l buffer
   ? 1?l BSA
   ? 5?l nanopure water
2. multiply with number of samples
3. label a 1500?l Ependorf tube for each sample
4. add restriction enzyme to master solution tube (1500?l Ependorf tube)
5. add buffer
   buffers are restriction enzyme-specific
6. add BSE if required
7. add nanopure water
   use calculated quantity plus 10% of calculated quantity of water
8. pipette 7?l of master solution into each reaction tube
9. add 3?l PCR product
10. incubate at required temperature overnight
   reaction conditions for restriction enzymes can be found in catalogue and in information sheet shipped with restriction enzymes
11. spin down (until ca. 3000rpm)
12. run 15?l on 10% acrylamide gel
13. store at -70?C

GELS

PREPARING AND RUNNING AGAROSE GELS

1. add 2g (for 1% agarose gel) Agarose (low EEO) to 200ml 0.5xTBE
2. add 6?l Ethidium Bromide
3. heat up in microwave for 3min (leave lid unscrewed)
4. transfer 40ml or 100ml (depending on required gel size) into Pyrex glass
5. cool for 1-2min under running water
6. pour into gel form
7. remove air bubbles in gel by moving gel comb
8. let sit for 20min
9. remove comb and stoppers
10. fill electrophoretic apparatus with 0.5xTBE buffer
   buffer can be reused many times but needs to be refilled since it tends to evaporate
11. submerge into electrophoretic apparatus with gel facing cathode
   gel surface has to be covered with buffer
8. put dye-dots on Parafilm
9. add 5?l PCR product (or as much as required) per dye dot
10. load DNA-dye mixture into gel grooves (pipetter set to 7?l) (Figure 6)
11. pipette 6?l (4?l if small gel) into gel buffer at anode end of gel

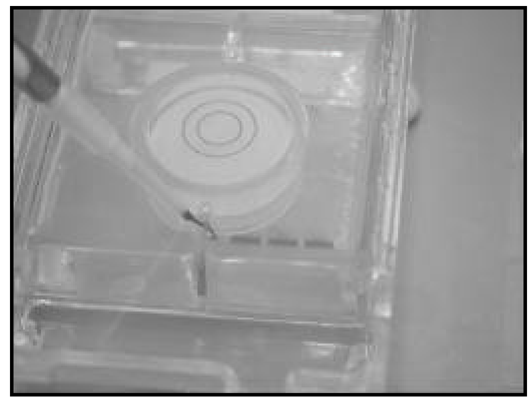
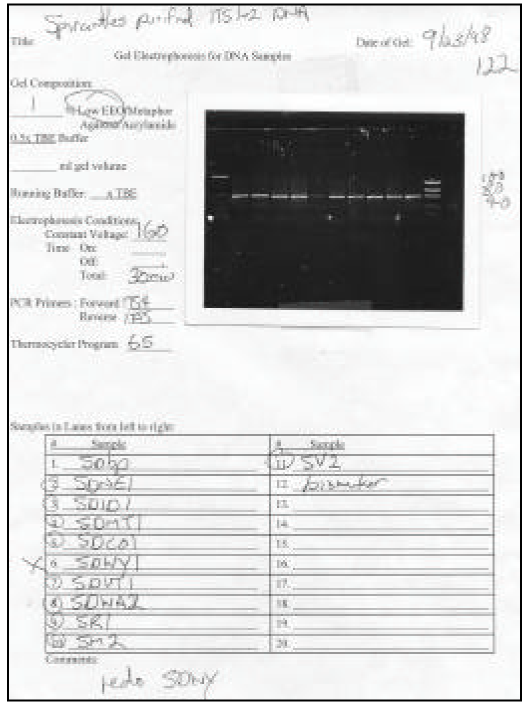


Figure 6: Loading agarose gel.

12. close lid
13. connect cables to transformer

14. set transformer to required voltage
    1% gel should be run at voltages between 160V and 170V
15. turn transformer on
    bubbles should emerge from cathode
16. fill in data sheet
17. run gel for 45 minutes or until dye line is near gel end
18. turn transformer off and disconnect wires from gel box
19. disconnect wires from transformer
20. remove gel with tray from apparatus (use glove)
21. slide gel onto Transilluminator
22. put on UV goggles ✍
23. place camera over gel
24. turn light off
25. turn Transilluminator on
26. expose film for 1sec at f3.5
27. remove Polaroid film from camera
28. wait 30sec (while shaking Polaroid)
29. open print and remove negative
30. tape positive onto gel data sheet
31. clean Transilluminator with Kimwipes


Title:
Date of Gel: 9/23/98
Gel Electrophoresis for DNA Samples

Gel Composition:
1 Low EEO Metaphor Agarose Acrylamide
0.5x TBE Buffer
_____ ml gel volume
Running Buffer: ___ x TBE
Electrophoresis Conditions:
Constant Voltage: 160
Time On:
Off:
Total:
PCR Primers: Forward
Reverse
Thermocycler Program: 65

Samples in Lanes from left to right:

# Sample	# Sample
1.	11. SV2
2.	12.
3.	13.
4.	14.
5.	15.
6.	16.
7.	17.
8.	18.
9. SR1	19.
10. SM2	20.

Comments:

Figure 7: Example gel sheet.

PREPARING AND RUNNING ACRYLAMIDE GELS

1. assemble gel forms
2. add gel ingredients while stirring (USE GLOVES!)

for 2 gels:

- ? 28ml nanopure water
- ? 5ml 10xTBE
- ? 17ml Acrylamide (for 10% gel)
- ? 350?l Ammonium Persulfate
- ? 200?l Photoflo
- ? 30?l Temed

  add Temed last since it causes the polymerization

3. stir for 30 sec
4. poor into gel form
   if gel is leaking lightly pipette at leakage and fill back in at top
5. insert comb
6. let sit for 1hr
7. fill 1xTBE into grooves
8. store in refrigerator
9. before using warm up to room temperature and remove comb
10. refill grooves with 1xTBE
11. load gel (with loading pipette tips)
12. attach cathode carrier
13. submerge gels in gel box
    box should be filled to about ½ with 1xTBE buffer
14. fill cathode-carrier with 1xTBE buffer until about 1cm above cathode
    buffer can be reused several times
15. plug into power source
16. turn power source on
17. select appropriate voltage (e.g. 350V)
    air bubbles should emerge from cathode
18. complete gel sheet

19. run for 1 ½ hrs or until dye line reaches gel bottom
20. turn power source off
21. disconnect wires
22. remove gel from gel-box (with gloves)
23. fill buffer from cathode carrier back into container
24. fill glass tray with 0.5xTBE buffer (ca. 1cm deep)
25. add 60?l Ethidium Bromide
26. mix well
27. remove plates from cathode-carrier
28. run spatula through top of gel plates to separate gels from each other
29. peel plates apart by inserting a free spacer
30. move gel into TBE-Ethidium Bromide bath
31. let sit for 10min
32. move gel onto transilluminator
33. place camera over gel
34. put on UV goggles ✍
35. turn off light
36. turn on transilluminator
37. take picture (f3.5/6-8sec)
38. remove Polaroid from camera
39. wait 30sec while shaking film
40. peel apart and remove negative
41. tape positive onto gel data sheet
42. move gel into gel basket
43. clean Transilluminator with Kimwipes
44. clean glass trays
45. clean glass plates (scrub under running water and rinse with sterile water)

FORENSIC WORK

SPECIES ID (MAMMALS)

GENERAL

1. run cytochrome b amplification with following primers (Kocher et al. 1989):
   - ? CytB(L14841) (5'-CCATCCAA
   - ? CATCTCAFCATFATFAAA-3')
   - ? CytB(15149) (5'-GCCCCTCAG
   - ? AATGATATTTGTCCTCA-3')
2. run restriction enzyme digest with following three restriction enzymes:
   - ? Msp I (5'…C/CGG…3')
   - ? Alu I (5'AG/CT…3')
   - ? Hae III (5'…GG/CC…3')
3. run on acrylamide gel
4. compare results with database to identify species
   (Examples: Table 1)

CERVIDS

1. run D-Loop amplification with following primers (Murray et al. 1995):
   - ? CERDL 1(5'-GGGTCGAAGG
   - ? CTGGGACCAAACC-3')
   - ? CERDL 2 (5'-TAATATACTGG
   - ? TCTTGTAAACC-3')
2. run restriction enzyme digest with following four restriction enzymes:
   - ? Msp I (5'…C/CGG…3')
   - ? Ase I (5'…AT/TAAT…3')
   - ? Hae III (5'…GG/CC…3')
   - ? Hha I (5'…GCG/C…3')
3. run on acrylamide gel
4. compare results with database to identify species (Figure 10)

DNA FINGERPRINTING

CERVID DNA FINGERPRINTING

1. use the following microsatellite primer pairs (DeWoody et al. 1995)
   - ? Cervid 1 forward/reverse
   - ? Cervid 3 forward/reverse
   - ? Cervid 4 forward/reverse
   - ? Cervid 14 forward/reverse
2. write out component quantities per tube for each primer pair
   - ? 1?l primer 1
   - ? 1?l primer 2
   - ? 5?l buffer
   - ? 4 ?l nucleotides (dNTP)
   - ? 0.5?l taq polymerase
   - ? 40?l nanopure water
3. proceed as described in "DNA Amplification" (steps 2. to 16.)

GENDER ID

MAMMAL GENDER ID

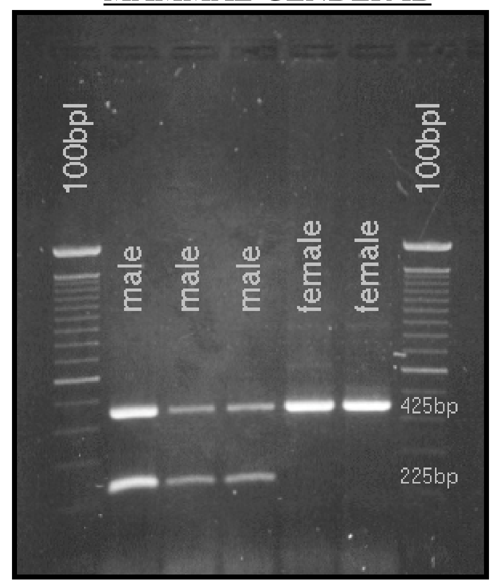


Figure 8: Gel with gender specific banding pattern for mammals.

1. add 2?l of each of the following primers and 92?l sterile water into one(!) 1500?l Ependorf tube:
   - ? Y53-3C (5'-CCCATGAACGCATTCATTGTGTGG-3')
   - ? Y53-3D (5'-ATTTTAGCCTTCCGACGAGGTCGATA-3')
   - ? P1-4EZ (5'-ATAATCACATGGAGAGCCACAAGCT-3')
   - ? P2-3EZ (5'-GCACTTCTTTGGTATCTGAGAAAGT-3')
2. write out component quantities per tube
   - ? 4?l gender ID primer mix
   - ? 5?l buffer
   - ? 4?l nucleotides (dNTP)
   - ? 0.5?l taq polymerase
   - ? 40?l nanopure water
3. proceed as described in "DNA Amplification" (steps 2 to 15)
4. run 15?l PCR product on 1% agarose gel at 160V-170V
   male DNA has two amplicons : female DNA has only one amplicon
5. store PCR product at -20?C

<u>AVIAN GENDER ID (Gallinaceous Birds)</u>

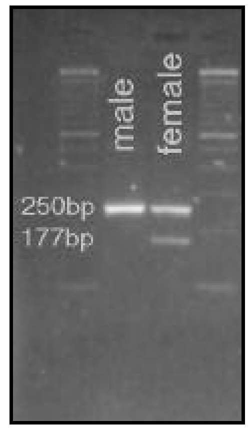


Figure 9: Gel with gender specific banding pattern for birds.

1. add 2?l of each of the following primers (D'Costa & Petitte 1998) and 96?l sterile water into one(!) 1500?l Ependorf tube
   - ? 5'-CAGGAAATGCCAGTTTATCG-3'
   - ? 5'-ATGTTTTGGGGGCAAAAATCC-3')
   - ? 5'-TTGGAGGAGGCTCTCTGACTGCTTTGCCCG-3')
   - ? 5'-CCAACGTTGATGGCTGGACGGATACCT-TTG-3'

   the primer concentration in the primer mix might have to be adjusted in order to achieve optimal quality of the PCR product
2. write out component quantities per tube
   - ? 4?l gender ID primer mix
   - ? 5?l buffer
   - ? 4 ?l nucleotides (dNTP)
   - ? 0.5?l taq polymerase
   - ? 40?l nanopure water
3. proceed as described in "DNA Amplification" (Steps 2 to 15)
4. run 15?l PCR product on 2.5% agarose gel at 115V-125V
   male DNA has one amplicons : female DNA has two
6. store PCR product at -20?C

Positive and negative controls should be run with all forensic PCR an RFLP reactions.

Table 1: Cytochrome B restriction sites of four restriction enzymes for common wildlife species

Species	Alu I	Hae III	Hinf II	Rsa I
Fallow deer, *Dama dama*	304	74, 233	161	none
White-tailed deer, *Odocoileus virginianus*	none	none	63, 161	none
Mule deer, *Odocoileus hemionus*	none	none	162	323
Red deer, *Cervus elaphus*	none	74, 233	44	238
Pronghorn, *Antilocapra americana*	304	74, 233	161	none
Bison, *Bison bison*	190	74, 233	161	326
Mountain goat *Oreamnos americanus*	none	74, 227, 233	44	none
Bighorn sheep, *Ovis canadensis*	none	74, 233	none	none
Wild Turkey, *Meleagris gallopavo*	none	74, 130, 190, 233	1, 161	249, 250
Ring-necked pheasant, *Phasianus colchicus*	none	74, 223	161, 278	154, 256
Human, *Homo sapiens*	none	233, 338	161	none

APPENDIX

THERMAL CYCLER PROFILES

Amp	Step	Number of Cycles	Denaturation		Annealing		Extension	
			Temp.	Duration	Temp.	Duration	Temp.	Duration
Cervid D-Loop Murray et al. 1995	Initial Denaturation	1	94?C	5min	54?C	30sec	72?C	2min
	PCR Cycles	30	94?C	15sec	54?C	30sec	72?C	2min
Cervid Microsatelitte DeWoody et al. 1995	Initial Denaturation	1	94?C	2min				
	PCR Cycles	35	94?C	30sec	58?C	30sec	72?C	30sec
	Final Extension						72?C	5min
Mammal Gender ID	Initial Denaturation	1	94?C	5min	60?C	45 sec	73?C	1min
	PCR Cycles	39	94?C	40sec	60?C	45sec	73?C	1min
Cytochrome B Unpublished data	Initial Denaturation	1	94?C	5min 30sec				
	PCR Cycles	35	94?C	45sec	48?C	45sec	72?C	1min
	Final Extension						72?C	10min
Bird Gender ID D'Costa & Petitte 1998	Initial Denaturation	1	94	1min 15sec				
	PCR Cycles	40	94?C	10sec	51?C	10sec	72?C	10sec
	Final Extension						72?C	5min
Fish D-Loop Bernatchez et al. 1992	Initial Denaturation	1	94?C	5min	50?C	45sec	72?C	2min
	PCR Cycles	34	94?C	40sec	50?C	45sec	72?C	2min
rDNA ITS I Szalanski et al. 1997	Initial Denaturation	1	94?C	2min 30sec				
	PCR Cycles	40	94?C	45sec	53?C	45sec	72?C	1min 30sec
	Final Extension						72?C	10min

Appropriate annealing temperatures for a certain primer can be determined with the help of the *Primer Calculator* on the following web site: http://www.res.bbsrc.ac.uk/biochem/oligos/input.html.
In general an initial denaturation (30sec-1min30sec) should be followed by 30-35 cycles of 94?C (45sec), annealing temp. (45sec), 72?C(45sec).

SOLUTIONS NEEDED

Solution	Recipe
1.0% Agarose, Low EEO (used for check gels) ✍ Cap tightly and store at RT ✍See under ‘Ethidium Bromide’	In a 250ml glass bottle, place 2.0g agarose and 200.0ml 0.5X TBE buffer and then aliquot 4.0?l 2.5mg/ml EtBr
10% Ammonium Persulfate ✍ store in refrigerator ✍ may cause irriation and sensitization of the respiratory system	Add 1g ammonium Persulfate Powder to 10ml H_2O.
0.5M EDTA, pH 8.0 ✍ Store at RT. ✍disodium-ethylenediaminetetra-acetate may cause respiratory and diggestive tract irritation as well as eye and skin irritation	Add 186.1g of disodium ethylenediaminetetraacetate . $2H_2O$ to about 600ml ddH_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (about 20g of NaOH pellets). *The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH. *Be very careful while adding NaOH to the solution when approaching pH 8.0 -- solution tends to change pH much faster the nearer it gets to 8.0. Pour the solution into a 1L graduate cylinder and bring volume up to 1L. (*Can make smaller volumes than this—ex. 200ml.) Dispense into aliquots (ex., 2 500ml, etc.) and sterilize by autoclaving.
10X Gel Dye Reagent Final Concentration in 10X Solon ✍ Store at constant -20oC. ✍see under 0.5M EDTA and 10X TBE	500?l glycerol 50% 400?l 0.5M EDTA 0.2M 2.5mg Bromophenol Blue (dye)0.25% About 5?l 1M Tris (i.e., however much is required to change the pH so that the color turns from green to blue) About 100?l sterile ddH_2O Approximately 1,000?l total volume Mix well by pumping and aliquot 1.0ml to as many sterilized 1.6ml centrifuge tubes as needed.
DNA extraction buffer ✍ Store at RT ✍see under 0.5M EDTA and 10X TBE	(100 mM EDTA, 100 mM NaCl, 100 mM Tris pH 7.5, 0.5% SDS, 200 ug proteinase K).

DNA Ladder (50bp, 100bp) ✍ Store in refrigerator	These come at a 1?g/?l concentration—extremely concentrated. Dilute 8.0?l of this stock in 392.0?l sterile ddH2O (1:50 dilution), aliquot 40.0?l gel dye, and mix by pumping several times. 7?l are used per lane on a check gel.
2.5mg/ml Ethidium Bromide (EtBr) ✍ Store at +4?C in the dark ✍powerful mutagen and moderately toxic. Gloves should be worn when working with solutions that contain this dye; check to see what regulations your lab may have regarding its disposal.	Add 0.25g (= 250mg) of ethidium bromide to 100ml of ddH2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. use about 6.0?l of this solution per 200.0ml of an agarose solution.
20uM working stocks of your primers ✍ Store at -20?C	In a 1.6ml centrifuge tube, aliquot 98.0?l sterile ddH2O, 98.0?l 1X TE, pH 8.0, and 4.0?l of 1mM primer storage stock (already mixed well by pumping). This gives 200.0?l of a 20?M working stock of your primer with a 0.5X TE concentration—an amount quickly used up with little chance of becoming degraded.

10X TBE (stock electrophoretic buffer) ✍ A precipitate forms when concentrated solutions of TBE are stored for long periods of time. To avoid problems, store the solutions at RT and discard any batches that develop a precipitate. ✍ boric acid may cause irritaion to the eyes and skin, it may also cause reproductive effects, TRIS can cause irritation to the eyes and skin and causes burns to the mouth and stomach when ingested	108.0 g Tris --- 0.89 M g Boric Acid --- 0.89 M ml 0.5 M EDTA, pH8.0 --- 0.02 M Dissolve the above reagents in 500-600ml ddH2O and then bring solution to 1L.
0.5X TBE (electrophoretic running buffer) ✍ store at RT ✍see 10X TBE	add 1L 10X TBE buffer to 19L of ddH2O. Mix well before using.
1X TE, pH 8.0 ✍ Store at RT. ✍see 10X TBE	Make up your needed volume of solution so that you have 10mM Tris . HCl, pH 8.0 & 1mM EDTA, pH 8.0 as your final concentrations. *For TE at a certain pH (ex., 8.0), use Tris-HCl at that certain pH. No matter at what pH you want the TE, use EDTA at pH 8.0. *We usually use TE at pH 8.0. Dispense into aliquots and sterilize by autoclaving.

"T" stands for Tris (or Tris-HCl) -- stabilizes DNA by buffering solutions very well
"B" stands for Boric Acid
"E" stands for EDTA (Disodium Ethylenediaminetetraacetate . 2H2O) -- chelates Mg+2 ions (i.e., takes them out of solution thereby preventing them from interacting with enzymes such as DNases and *Taq* Polymerase).

**Molarity (M) is a concentration (i.e., moles per liter), while moles (m) is an amount.

** **ddH2O** refers to distilled, dionized water and sometimes to double distilled water, which are two different things, but both appear to work equally well in the various protocols/recipes. Store at room temperature (RT).
*Remember, 1.0g of solute in 100.0ml of liquid makes a 1.0% solution.

LITERATURE CITED

Bernatchez, L., R. Guyomard, and F. Bonhomme. 1992. DNA sequence variation of the mitochondrial control region among geographically and morphhologically remote European brown trout, *Salmo trutta*, populations.Molecular Ecology 1:161-173.

D’Costa, S. and J.N. Petitte, 1998. Sex identification of turkey embryos using a multiplex polymerase chain reaction. Poultry Science 77:718-721.

D’Woody, J.A., R.L. Honeycutt and L.C. Skow, 1995. Microsatellite markers in white-tailed deer. J. Hered. 86:317-319.

Kocher , T.D., W.K. Thomas, A. Meyer, S.V. Edwards, S. Paabo, F.X. Villablance, and A.C. Willson. 1989. Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conservbed primers. Proc. Nat. Acad. Sci. 86: 6196-6200.

Szalanski, A.L., D.D. Sui, T.S. Harris, and T.O. Powers. 1997. Identification of cyst nematodes of agronomic and regulatory concern with PCR-RFLP of IS1. J. Nematol. 29:255-267.