Canine Oxytocin Receptor Genotypes

Overview (See handout from last week regarding Oxytocin Receptor and Dog behavior)
With the use of DNA sequencing techniques, researchers have identified various common OXTR genotypes in dogs. There are three known sites where the DNA sequence is different in different genetic variants (Figure 1). These are called single nucleotide polymorphisms (SNP) because they each involve only a single nucleotide base. None of the identified canine SNPs alter the structure of the protein and it is not known if or how they impact the level or location of OXTR gene expression. While there are some functional studies that support a functional role for oxytocin in affection seeking behavior in dogs (Person et al 2017) the data suggesting that the OXTR gene and these polymorphisms specifically impact dog behavior are purely correlational. In this lab we aim to add to this correlational data to determine if one of these SNPs is associated with the behaviors on which we tested Reed dogs last week. We will use PCR amplification and Restriction Fragment Length Polymorphism analysis to determine the OXTR genotype for each dog.

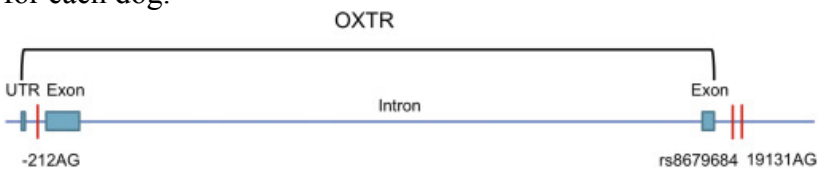


Figure 1. Oxytocin receptor gene & 3 SNP sites associated with different aspects of dog behavior. 2 are named by their location relative to the start site and the third SNP bears a historical name.

Restriction fragment length polymorphisms (RFLPs)

Restriction sites (Fig. 2) are short (usually 4 – 6) nucleotide sequences that can be cut by prokaryotic **restriction enzymes**, which cleave DNA at specific **recognition sequences** called restriction sites. These restriction enzymes are naturally-occurring in bacteria and archaea and are used by the prokaryote as a form of protection against viral infection. Upon viral infection, the prokaryotic restriction enzymes will cut the viral DNA wherever the recognition sequence exists. Many different restriction enzymes have been identified from many different bacteria, each with a unique recognition sequence. Biologists have exploited these in recombinant DNA technology because they allow the cutting and pasting of DNA sequences from one place to another, analogous to cutting and pasting a phrase or sentence in a word processor from one part of a paragraph to another. When a SNP changes whether the DNA sequence contains a restriction enzyme recognition sequence, restriction enzyme "digestion" can also be used in "DNA fingerprinting", a technique that is applied to forensics and paternity testing. The SNP at position 212 bp upstream of the oxytocin receptor gene start site (-212) contains an A in some genotypes and a G in others. The A is part of the recognition sequence for the restriction enzyme Hpy991 whereas the G creates a sequence that is not cut by this enzyme (**Fig. 3**).

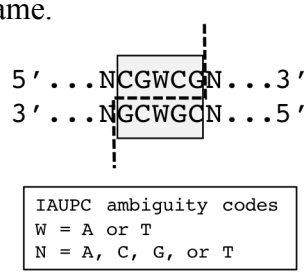
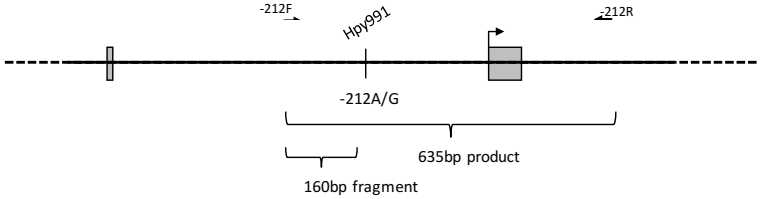


Figure 2. DNA recognition sequence and cut sites for the restriction enzyme Hpy991. N indicates that any nucleotide can be present in that location for effective enzyme function. W indicates that either A or T can be part of the recognition sequence. Dotted line indicate the location of the double-stranded cut and resulting overhang made by the restriction enzyme.

Figure 3. Map of the Canine Oxytocin Receptor gene. An A/G SNP that is 212 bp upstream from the start site of the gene creates an RFLP for the Hpy991 restriction enzyme. Half arrows indicate the forward and reverse primers that create a 635 bp PCR product. There is an additional 3' exon not shown several kb away.



Before lab, read this handout,
If you are unfamiliar with PCR watch this animation to review the protocol:
http://www.dnalc.org/view/15475-The-cycles-of-the-polymerase-chain-reaction-PCR-3D-animation.html

PCR: Each student pair materials:

- ddH20
- 5M Betaine (Sigma #B0300-1VL)
- REDExtract-N-Amp PCR Reaction Mix (Sigma #R4775).
- Canine OTXR primers (IDT):
- Fwd-primer "-212F": 5'-CCATTGGAATCCGCCCCCT-3'
- Rev-primer "-212R": 5'-CACCACCAGGTCGGCTATG-3'
- 2 PCR 8 strip-tube and lids
- Dog DNA (extracted previously)
- Homozygous A allele positive control (from Aldo)
- 100 µl pipette
- 10 µl pipette (ok to use 20 µl and 200 µl instead)
- pipette tips
- ice bucket

RFLP analysis: Each student pair materials:

- Restriction enzymes Hpy991
- Restriction enzyme buffer (NEB cutsmart)
- Gel electrophoresis supplies dH20
- 1X TBE,
- gel box,
- power supply,
- agarose,
- SybrSafe,
- 50 bp DNA ladder (NEB NO556S)
- Purple 6X loading dye (NEB #B7025)

Lab equipment:

- Thermocycler
- Ice cooler with Ice
- 37°C waterbath

Carefully record information as you work. This includes the volumes and the times involved. Remember: Make a note when your procedure deviates from the protocol (e.g., spilled solution, sneezing into a tube, leaving it too long at one step, etc.). *One of the biggest errors you can make in science is not making note of errors.*

PCR amplify the OXTR -212AG SNP Work in groups of 2.

1. **Keep reagents on ice while setting up reactions and wear gloves.**
2. Each group of perform a PCR reaction for each of 4-6 dog DNA samples as well as a positive control sample (a homozygous A genotype) and one negative control sample.
3. Label a strip-tube on the side just below the lid with a fine point sharpie. Include enough information to identify it as yours and what is in each tube. (your initials & dog ID).
4. Using the following recipe, create a PCR cocktail for the number of reactions you will run.

20 µl reaction volume

	____ # reactions + 10% (i.e. for 8 reactions multiply each reagent volume by 8.8)
4 µl Betaine 5M	
1 µl -212AG-R 10 uM	
1 µl -212AG-F 10 uM	
10 µl Red Extract and Amp Polymerase	

Note: betaine is not used in standard PCR. It is an additive for high in GC content regions.

5. **Flick to mix** and spin down.
6. Aliquot 16 µl of the PCR cocktail into each well of the strip-tube.
7. Add 4 µl gDNA to each tube, 4 µl of positive control and 4 µl of water to the negative control.
8. Put the cap-strip on the strip-tube securely.
9. Flick the strip-tube to mix and spin.
10. Placing your strip-tube in the rack in the ice tub next to the thermocycler.
11. When the class is ready, we will load these into the PCR machine and start the cycling.

    The thermocycler program will be:

    94°C –3 minutes (initial denaturation)

    35 cycles of:

    94°C for 30 seconds (denaturing)

    56°C for 30 seconds (annealing)

    72°C for 1 minute (extension)

    72°C for 5 minutes (final extension)

    12°C indefinitely (final hold)
12. Clean your lab space: dispose of gloves, used tips, tubes, etc.,
13.

While you wait for the PCR (day2: go to gel pouring and come back to this while the gel is running)

14. Make a sketch of your anticipated results for a dog that is homozygous for the A allele, one that is homozygous for the G allele and one that is heterozygous at the -212AG locus.
15. Make a sketch of your anticipated results for a dog that is homozygous for the A allele but the restriction digest is "incomplete" (only some of the DNA strands are cut).
16. How will you be confident that a heterozygous dog is really heterozygous and not simply a restriction digest that did not go to "completion"?
17. Consider what figures you might make to present the behavior and genotype data. Sketch a few in your notebook. What will be on each axis? how will the data be grouped? Would a table present the data better? What questions can you answer with the data?
18. Read Kis et al 2014 or Persson et al 2017 (on the course website) and consider what you predicted results are. (please feel free to find your own sources as well)
19. Go to the class "manuscript" on google drive and contribute to our paper.

Identify genotypes by RFLP pattern

Set up restriction digests:

1. Remove your PCR products from the thermocyler.
2. Label a 0.2 strip-tube for the restriction digest reactions (follow format from PCR)
3. **Keep reagents on ice while setting up reactions:**
4. Using the following recipe, create a digest cocktail

	____ # reactions + 10%
7 µl ddH2O	
2 µl 10X NEB cutsmart buffer	
1 µl Hyy991 restriction enzyme	

1. **Flick to mix** and spin down.
2. Aliquot 10 µl of the Restriction digest cocktail into each tube in the strip.
3. Add 10 µl PCR product to the appropriate labeled tube (including controls).
4. **Flick to mix** and spin down.
5. Place the tube in the water bath set to a **37°C** and fill in the class digest map on the counter to indicate where your tubes are.
6. Incubate tubes 4 hours. Start _____ Stop _____
7. Fill in the information/labeling sheet.
8. Save the unused PCR reactions in a 96well rack in the freezer, with a lid and the information/labeling sheet neatly folded. (Your digested samples will be added to these racks when they are done)

Prepare gel:

While waiting for the digest, make a 2% agarose gel for your group. *Rule of thumb: In order to make a 1.0% gel, you would measure out 1 g of agarose powder on the balance, and add it to a beaker containing 100 ml of TBE buffer.*

9. You need to make about 60 ml of your 2% agarose solution to pour two gels of the size we need. Do the calculations to figure out how much agarose (grams) you need to add to 60 ml of buffer to get the right concentration. Record your recipe in your lab notebook.
10. Weigh out the appropriate amount of agarose and add it to the appropriate amount of buffer in a glass flask that is much larger than the volume you are working with—swirl it to mix it, but notice that the agarose won't dissolve. You must microwave it briefly (set for 1 minute) on High to bring it to a boil to dissolve the agarose. **Keep an eye on the gel *while* microwaving**, as you want it to come to a rolling boil for ~20 sec. Keep in mind that if you microwave the mixture too long, the buffer will evaporate, resulting in a gel that is a higher concentration than you calculated originally.
11. Carefully remove your flask from the microwave once the agarose has dissolved—it should be completely clear after this step with no chunks or powder remaining in the flask. **It will be hot!** Use mitts. Let the solution cool slightly before doing anything else. If you pour boiling hot agarose into a plastic mold, it will likely warp or break the mold, and it can leak.
12. After removing the agarose from the microwave, add 6 µl SybrSafe stock solution (on front bench @ room temperature). This binds to your DNA and fluoresces in UV light, allowing you to visualize DNA bands in the gel.
13. Once the solution has cooled so that you can touch the flask with bare hands (but not so much that the agarose solution has started to solidify), it is ready to pour into the mold. We will need to share casting trays. **Pour it slowly** to prevent bubbles from forming. **Rinse out the flask immediately with warm water followed by distilled water**, as the agarose will solidify in there and be hard to clean.
14. Once you have poured the gel into the mold, **select a 9-tooth comb** and place it in the end of the tray (not in the middle). This comb will create enough wells for 8 samples and 8 digested samples plus a ladder on each gel.
20. Let your gel cool fully undisturbed (at least 15 min on the bench top). While your gel is cooling, draw a picture in your lab notebook of the gels you are planning to load. Put the 50 bp ladder (**Fig. 4**) on one side and load the undigested PCR product next to the corresponding digested PCR product.
21. During the digest and gel cooling, make a sketch of your anticipated results for a dog that is homozygous for the A allele, one that is homozygous for the G allele and one that is heterozygous at the -212AG locus.
22. Make a sketch of your anticipated results for a dog homozygous for the A allele but the restriction digest is "incomplete" (only some of the DNA strands are cut).
23. How will you be confident that a heterozygous dog is really heterozygous and not simply a restriction digest that did not go to "completion"?

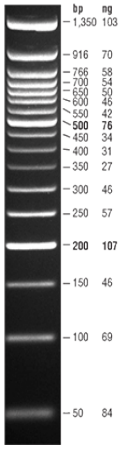


Figure 4. Quick-load Purple 50 bp DNA Ladder. This pre-mixed molecular weight marker contains one tracking dye. The ladder consists of a proprietary plasmids, which are digested to completion with appropriate restriction enzymes to yield 17 bands for use as molecular weight standards for agarose gel electrophoresis. This digested DNA includes fragments ranging from 50 to 1,350 base pairs. The 200 and 500 bp bands have increased intensity to serve as reference points.

Prepare your digested samples and load the gel:

15. Once the gel is cooled, remove the comb and set the gel on its tray into the gel box with the wells at the BLACK electrode end. Pour ~275 ml TBE gel running buffer on top to completely cover the gel (you might want to note this setup in your notebook for future reference).
16. Retrieve digested and undigested samples from the freezer, add 4 µl of 6X purple gel loading buffer (in refrigerator) to each 20 µl reaction. Also measure into a fresh tube 12 µl of 50 bp ladder pre-mixed with purple gel loading buffer (front bench). Spin down you samples.
17. Load 15 µl into a well on the gel for each digest, plus the 12 µl of the 50 bp ladder (**Fig. 4**). Follow your gel-loading map in your lab notebook.
18. Electrophorese the gel at 95 volts for about 50 minutes. Run until the faster-migrating dye runs to about 1 cm from the end, but NOT off the gel.
19. Place gel on EZ Imager to photograph the gel. *Your lab instructor will help you generate a digital and printed copy of your gel, which you will need to determine your genotypes.*
20. **Examine the image to determine each dog's genotype** based on restriction fragment sizes in your sample. Keep in mind that any heterozygote will have two fragment sizes. Also note that only two known genotypes are typically found in dog populations. Therefore, information from the two reactions should allow you to determine your precise diploid genotype.
21. Enter the data in the class dataset. Enter the genotype (**A/A A/G G/G**) if the results are clear. If the PCR failed, enter **NA**. If the results are ambiguous (incomplete digest), enter or **G/?** .
22. Complete the information on the information/labeling sheet to return.
23. Return to "While you wait for the PCR" section above where you will work on the class manuscript until your own PCR reactions are complete and ready to digest.

If the class works to complete a solid manuscript that can be distributed to all dog owners volunteers for this study, there will be no individual lab writeups.