# A GENETIC COMPARISON OF PITUITARY *PARS INTERMEDIA* DYSFUNCTION POSITIVE & NEGATIVE EQUINES AT DBH, TO AID IN THE DEVELOPMENT OF GENETIC DIAGNOSTIC PROTOCOLS.

by

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# ABSTRACT

Pituitary pars intermedia dysfunction (PPID) is the most common endocrine disorder of horses and ponies, and is a progressive neurodegenerative disease resulting from a loss of dopaminergic periventricular neurons that innervate the pars intermedia. Currently, diagnosis is made by a combination of clinical signs and multiple endocrine hormone tests. Using DNA extracted from equine hair follicles, the dopamine beta-hydroxylase gene (DBH) was studied as a candidate gene for association with PPID. Preliminary research indicates high levels of variation in all animals sampled, with no correlation to PPID, indicating further research must be conducted to determine the genes associated with this disorder, as PPID is likely to be influenced by multiple genes and the environment, much like other neurodegenerative disorders.

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*This thesis is dedicated to my horses, Sunday Best and Besst A Bar.*

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CHAPTER 1

# A GENETIC COMPARISON OF PITUITARY *PARS INTERMEDIA* DYSFUNCTION POSITIVE & NEGATIVE EQUINES AT DBH, TO AID IN THE DEVELOPMENT OF GENETIC DIAGNOSTIC PROTOCOLS

## INTRODUCTION TO PITUITARY *PARS INTERMEDIA* DYSFUNCTION

Pituitary *pars intermedia* dysfunction (PPID) is the most common endocrine disorder of horses and ponies.[1-2] Once referred to as Cushing's disease, due to its apparent similarity to Cushing's disease in humans and dogs, the acronym PPID is preferred by veterinarians and researchers as it more accurately reflects the pathophysiology. In humans and dogs Cushing's disease is characterized by an increase in serum cortisol concentration, and can occur due to exogenous or endogenous influences that affect the levels of the hormone cortisol in the body.[3] This characteristic is not found in horses with PPID. Equine pituitary *pars intermedia* disorder is a progressive neurodegenerative disease resulting from a loss of dopaminergic inhibition and causing a decrease in dopamine production in the *pars intermedia*.[2] Generally affecting older animals, there is a disturbing trend of younger animals developing PPID. Symptoms are gradual and progressive, often resulting in diagnosis of this disorder being delayed. Diagnosis is made by a combination of clinical signs and endocrine hormone testing, although repeated testing is often required to definitively diagnose the horse and, by then, this disorder is already reasonably advanced. It is likely that both genetics and

environment influence the possibility of developing PPID, much like other neurodegenerative disorders[2], such as Parkinson's disease (PD). The objective of this research is to identify a genetic marker in PPID positive horses to aid in the early diagnosis of this disorder in suspected cases, or that could be incorporated into breeding and performance programs to offer early screening.

**The equine pituitary gland and the function of the equine pituitary *pars intermedia***

The equine pituitary gland is suspended ventral to the hypothalamus by the infundibular stalk and consists of four distinct lobes; the *pars digitalis*, *pars nervosa*, *pars tuberalis*, and *pars intermedia* (Figure I.1).[1,2] The *pars digitalis* secretes growth hormones, follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, prolactin, and endorphins, and is regulated by specific hormones released by the hypothalamus and transported to the pituitary by the pituitary portal system, or the hypophyseal portal system.[1,2,4] The *pars nervosa* stores and secretes oxytocin and vasopressin, which are synthesized in the hypothalamus.[1,2] Little is known about the function of the *pars tuberalis* in equines, although in other animals it directs the seasonal output of reproductive hormones.[2] The *pars intermedia* is comprised of one endocrine cell, the melanotrope, which produces polypeptide pro-opiomelanocortin (POMC) which undergoes extensive processing by prohormone convertase (PC) 1 and 2 to yield many bioactive peptides such as alpha-melanocyte stimulating hormone (α-MSH) and beta-endorphins, as well as adrenocorticotropic hormone (ACTH) and corticotropin-like intermediate peptide (CLIP).[2] These peptides continue to undergo extensive post-

translational alterations, which alters the molecules' activity (Figure I.2).[2] There is also an abundance of receptors with which the POMC peptides can bind to, expressed in different anatomical locations and exhibiting different affinities for the numerous peptides produced.[2] Only about 2% of circulating ACTH is derived from the *pars intermedia* in normal horses; physiological production of ACTH is in the *pars digitalis*.[5]

Peptide production by melanotropes in the *pars intermedia* is controlled by hypothalamic and systemic neurochemicals, such as dopamine and thyrotropin.[2] It can be presumed that other chemicals are involved in regulating the *pars intermedia* based on studies conducted with other animals. In rats and amphibians, serotonin, GABA, and norepinephrine act as regulators in the brain, however, not much is known about other *pars intermedia* regulators in horses.[2]

Dopaminergic neurons directly innervate the *pars intermedia* from the hypothalamus, and terminate on the melanotropes, where dopamine binds to the inhibitory D2 dopaminergic receptors and acts as regulator of hormone synthesis and an inhibitor of cell division.[2] It should be noted that the products of POMC are exceptionally pleiotropic in function, and the three principal products are α-MSH, β-endorphin, and CLIP.[2] When ACTH is cleaved at the n-terminal region the product is α-MSH, and it plays a role in obesity, metabolism, inflammation, and stress.[2] When ACTH is cleaved at the c-terminal region the product is CLIP; unfortunately at this time little is known about the role of CLIP in the equine *pars intermedia*, although recent research in rodents has begun to link it to stimulation of the release of insulin.[2] β-endorphin is a powerful

endogenous opioid mu receptor agonist and acts in analgesia and the reduction of pain-associated inflammation.[2] Activity of the *pars intermedia* has been shown to have a strong seasonal rhythm leading to increased activity and output as days shorten, resulting in a higher plasma concentration of *pars intermedia* hormones through the months of August – October.[2]

**The mechanism of pituitary *pars intermedia* dysfunction**

PPID is linked to increased activity and size of the *pars intermedia*; necropsy reveals enlarged pituitary glands in horses with PPID, which is caused by hypertrophy or hyperplasia of the *pars intermedia*.[2] A single adenoma, or many microadenomas, are often present and can cause compression of the adjacent structures.[2] PPID has recently been re-categorized as a neurodegenerative disease, characterized by a loss of inhibitory dopaminergic input to the *pars intermedia.*[7] The degeneration of the dopaminergic neurons results in a decrease in dopamine production; the loss of this negative control results in the overproduction of POMC-derived peptides including ACTH, CLIP, α-MSH, and β-endorphin.[6] Researcher Dr. Dianne McFarlane, of Oklahoma State University, states that dopaminergic neurons appear normal in number and in dopamine biosynthesis in the *substantia nigra* of PPID positive equines. Additionally, her research has shown blood concentrations of dopamine were increased when measured by High Performance Liquid Chromatography (HPLC) in a small number of horses.

Horses with PPID display a significant reduction in dopamine in the *pars intermedia* tissue and loss of dopaminergic periventricular nerve terminals and cell

bodies.[2] The precise origin of this neurodegeneration has yet to be identified, although evidence has linked oxidative stress to the damage caused to the dopaminergic neurons; it is still unknown if oxidative stress is the cause or the result of this neurodegeneration.[2] Histological examination of deceased horses with PPID revealed the accumulation of 3-nitrotyrosine in the dopaminergic periventricular nerve terminals, evidence of oxidative stress.[7] Horses with PPID showed 16 times more 3-nitrotyrosine than in young controls, while aged horses without PPID displayed 7 times more 3-nitrotyrosine.[7] It has been demonstrated in other species, including humans, that the accumulation of oxidative damage with age contributes to the age-related risk of neurodegeneration.[7]

The pathogenesis of human neurodegenerative diseases has been linked to the neuronal accumulation of misfolded proteins, such as in Parkinson's disease, a dopaminergic neurodegenerative disease.[2] The protein that accumulates in Parkinson's disease is α-synuclein, and this aggregation causes disrupted cell function and cell death.[2] Dr. McFarlane has confirmed that, in horses with PPID, α-synuclein concentration and expression is increased in the *pars intermedia* and is peroxynitrite modified, a similar finding in patients with Parkinson's disease (Figure I.3).[2,7]

Researchers of neurodegenerative disorders acknowledge that Parkinson's disease is a multisymptom disorder, affecting not only the *substantia nigra*, but also the *locus ceruleus*.[8] The initiating factors of PD are hard to pinpoint, as it is an age-associated neurodegenerative disorder characterized by the loss of dopaminergic neurons in the *substantia nigra pars compacta* and the presence of Lewy bodies in both the *substantia*

*nigra* and the *locus ceruleus*.[7] Like PPID, the loss of neurodegeneration of the dopaminergic neurons results in a loss of inhibitory dopamine[7]; the difference lies in the location of the neurodegeneration. In PPID it occurs in the *pars intermedia* and in PD it occurs in the *substantia nigra*.

PD pathology involves the serotonergic and cholinergic systems as well, and results in neuronal loss in the *locus ceruleus*, the major group of noradrenergic neurons in the brain.[8] Studies suggest the loss of noradrenergic neurons may precede the loss of dopaminergic neurons, and this loss is as great as, if not more extreme, than that of the dopaminergic neurons.[15] The loss of these noradrenergic neurons appears to amplify both the motor symptoms of PD and the degeneration of dopaminergic neurons.[8] Current research has provided conflicting results regarding the effect of norepinephrine on dopaminergic neurons; some suggest norepinephrine is neuroprotective, and that degeneration of noradrenergic neurons sensitizes dopaminergic neurons to damage,[8,15] while others state that norepinephrine has no direct effect on dopaminergic neuron survival.[14] What researchers can agree on is that further examination of noradrenergic neuronal loss, and the effect of norepinephrine on dopaminergic neurons, is needed to understand the progression of PD and improve existing treatments. Currently, no information is available on the effect of norepinephrine in horses with PPID; however, it stands to reason given the similarities between PPID and PD that consideration should be given to the possible loss of noradrenergic neurons here as well. At this time, no

substantive research has been done using PPID as a systemic model for PD, although Dr. McFarlane has begun to explore this topic.[7]

**Clinical signs of PPID**

The progressive nature of PPID makes diagnosis difficult, as early signs tend to be nonspecific and require in-depth diagnostic testing, although age is a primary risk factor.[2] It is speculated by many veterinarians that subclinical PPID could be present for months to years before a positive diagnosis is made.[5] Signs include hypertrichosis, depression/lethargy, loss of performance, increased appetite, polydipsia, polyuria, laminitis, chronic hoof abnormalities, wasted topline or pot belly, bulging supraorbital fat, excessive or decreased sweating, infertility, and susceptibility to secondary infections.[5,6] While laminitis is considered to be the most serious complication of PPID (Figure I.4) not all horses will develop it; many horses who are otherwise asymptomatic for PPID can develop laminitis and chronic hoof complications.[5] PPID positive equines tend to develop a specific appearance as this disorder progresses (Figure I.5), although treatment can help diminish some of the signs.

**Current diagnostic techniques and treatments**

Diagnosis of PPID consists of the presence of clinical signs, testing endogenous hormone levels, and dynamic endocrine testing. [5,9] Horses with indefinite results or in what is considered a "grey zone", which is clinically normal horses with slightly elevated ACTH, are recommending to undergo resampling or thyrotropin-releasing hormone (TRH) stimulation test measuring ACTH to improve sensitivity.[9] Due to the seasonal

rhythm of the *pars intermedia* seasonally adjusted reference ranges must be used depending upon the time of year testing occurs to ensure accuracy.[9,10]

Measurement of plasma ACTH is the current test of choice among veterinarians. Concerns regarding specificity and sensitivity of this test have been reduced with the development of seasonally adjusted reference ranges. ACTH rises in the autumn months in both PPID positive and normal horses, though, the increase is greater in PPID positive horses.[5,9] There are several limitations to plasma ACTH testing, including stress level, biological variation, feeding, and stability. It is advised to avoid testing around stressful stimuli, and in situations where the horse is in pain to retest to confirm the increase in ACTH is due to PPID and not stress.[10]

The TRH stimulation test was originally designed to measure cortisol levels, but recent findings demonstrate that measuring plasma ACTH to be superior in PPID testing.[9] TRH administration raises ACTH and cortisol levels in both normal and PPID positive horses; again the affect is greater in horses with PPID.[10] Plasma ACTH peaks roughly 10 minutes after TRH injection before gradually decreasing to the horses' normal level, so endogenous ACTH levels are measured at 2, 10, and 30 minute intervals.[9,10] This test is subject to most of the same limitations as the plasma ACTH test, and, additionally, there are no seasonal reference intervals so results cannot be reliably read during the autumn season.[10]

Once regarded as the "gold standard" in PPID testing and diagnostics, the overnight dexamethasone suppression test (ODST) has been shown to be less reliable

than plasma ATCH or TRH stimulation in detecting early disease.[5,9,10] With no seasonally adjusted references, the expense of two hormone assays, and the risk of exacerbating or inducing laminitis, the limitations of the ODST has caused veterinarians to stop recommending it for PPID diagnosis.

Currently being researched it the α-MSH assay, though it is not yet commercially available. α-MSH plasma levels tend to be higher than ACTH levels in PPID positive horses, and it has been suggested that α-MSH levels increase earlier in the disease progression and may be a more sensitive diagnostic test.[9]

In horses diagnosed with PPID it is advised to test insulin levels, as hyperinsulinaemia has been established as a cause of laminitis.[5,9,10]

There is no cure for PPID, only treatment and management. Pergolide mesylate, an ergot-derived D2-dopamine agonist, is the drug of choice for treatment of PPID.[9,11] Cyproheptadine, a serotonin antagonist, was once widely used but has since been proven relatively ineffective in PPID treatment.[11] Pergolide mesylate replaces dopamine and reestablishes dopamine inhibition, resulting in the normalization of pituitary hormone levels and often an elimination of clinical signs.[9,11] Management of PPID positive horses is essential, and includes general good husbandry, clipping of excessive hair growth in warm climates, careful dietary management, good farriery and monitoring for laminitis, and routine dental and veterinary checkups.

## METHODS

### Sample Collection

Hair samples were collected from either the mane or tail of the horse based on the owner's preference and then stored at room temperature in individual donation bags out of direct light. Blood samples were collected in heparin tubes from the jugular during routine yearly examinations by board certified veterinarians and stored at 4°C (if the sample was to be immediately analyzed) or at -20°C (if analysis was to be delayed). All samples were donated by participating veterinarians, Panhandle Safe Hayven Equine Rescue, and horse owners from January 2015 – April 2016. All participants and horse owners were given the option of signing a "Consent to Participate in Research" form before donating to this research. Dr. Jeff Young donated two blood samples and two hair samples collected from participating clients; only the hair samples were used in this study. Dr. Katie Dickenson donated blood samples collected from three horses from a participating client; only the PPID positive sample was used in this study. Dr. Amber Reiman donated fifteen hair samples and twelve blood samples collected from her participating clients; only the hair samples were used in this study. Panhandle Safe Hayven Equine Rescue donated eleven hair samples, all of which were used in this study. Countless horse owners, riding centers, and two other veterinarians, who requested to have their names withheld, donated hair samples for use in this research. Samples were obtained from across Texas, although many horses originated from various states across the U.S.

Samples were collected from a wide variety of breeds to better determine the heterozygosity of the dopamine beta-hydroxylase (DBH) gene on chromosome 25. Breeds and quantities used in this study were American Quarter Horse (20), Appendix Quarter Horse (1), Hackney (1), Gypsy Vanner (2), Thoroughbred (5), Pony (1), American Paint (4), Tennessee Walker (2), Mustang (2), Arabian (4), Hackney/Arabian (1), Thoroughbred/Paint/Quarter Horse (1), Quarter Horse/Paint, Thoroughbred/Quarter Horse/Irish Sport Horse (1), and Irish Sport Horse (1). Of these, four were diagnosed PPID positive and six are awaiting veterinary diagnosis for their PPID symptoms. The PPID positive/possible breeds and quantities were Arabian (1), Appendix Quarter Horse (1), and American Quarter Horse (7). All samples were entered into an Excel spreadsheet for proper cataloging (Table I.1).

**DNA Extraction**

DNA extraction of hair was performed using a modified DNeasy protocol (QUIAGEN Corporation, Valencia, CA) for purification of total DNA from animal tissues (spin-column protocol). Modification concerned adjusting the amount of elution buffer for tissue based off the quantity of DNA present in the final wash when visualized in a 1% agarose gel following electrophoresis. 15 hair follicles (2-5 mm in length) were placed in a 1.5 ml microcentrifuge tube and 180 µl Buffer ATL was added. Grinding or disruption of the follicles was not necessary. 20 µl proteinase K was added and vortexed for 10 seconds before incubation at 56°C for 3 hours. After incubation the samples were vortexed for 10 seconds before 200 µl Buffer AL was added. Samples were vortexed for

10 seconds, 200 µl 99% ethanol was added, and samples were vortexed again for 10 seconds. The mixture (including the precipitate) was pipetted into a DNeasy Mini spin column, placed in a 2 ml collection tube, and centrifuged for 1 minute at 8000 rpm. The flow-through and collection tube were discarded, the spin column was placed in a new collection tube, 500 µl Buffer AW1 was pipetted into the spin column and it was centrifuged for 1 minute at 8000 rpm. The flow-through and collection tube were discarded, the spin column was placed in a new collection tube, 500 µl Buffer AW2 was added to the spin column and it was centrifuged for 4 minutes at 13.3 rpm. Again, the flow-through and collection tube was discarded. The spin column was placed in a 1.5 ml microcentrifuge tube and 50 µl Buffer AE was pipetted directly onto the DNeasy membrane and incubated at room temperature for 1 minute before being centrifuged for 1 minute at 8000 rpm. Samples were stored at 4°C for immediate use, or at -5°C for future use.

DNA extraction of blood was performed using a modified DNeasy protocol (QIAGEN Corporation, Valencia, CA) for purification of total DNA from animal blood (spin-column protocol). Modification concerned adjusting the amount of elution buffer for nonnucleated blood based off the quantity of DNA present in the final wash when visualized in a 1% agarose gel following electrophoresis. 100 µl anticoagulant-treated whole blood was pipetted into a 1.5 ml microcentrifuge tube with 20 µl proteinase K and 100 µl PBS. 200 µl Buffer AL was added to the tube, vortexed for 10 seconds, and incubated at 56°C for 10 minutes. 200 µl of 99% ethanol was then added to the tube and

vortexed for 10 seconds. The mixture was pipetted into a DNeasy Mini spin column, placed in a 2 ml collection tube, and centrifuged for 1 minute at 8000 rpm. The flow-through and collection tube were then disposed of, the spin column was placed in a clean collection tube, and 500 µl Buffer AW1 was added to the spin column and centrifuged for 1 minute at 8000 rpm. The flow-through and collection tube were disposed of, the spin column was placed in a clean collection tube, and 500 µl Buffer AW2 was added to the spin column and centrifuged for 4 minutes at 13.3 rpm. The flow-through and collection tube was again disposed of, the spin column was placed in a sterile 1.5 ml microcentrifuge tube, and the DNA was eluted with 50 µl Buffer AE and allowed to incubate at room temperature for 1 minute before centrifuging for 1 minute at 8000 rpm. Samples were stored at 4°C for immediate use, or at -5°C for future use.

**PCR amplification and purification**

Five primer pairs were chosen based off their relationship to equine dopamine receptors, DRD2, DRD4, DRD1, SLC66A3, and DBH. Of these primer pairs, only one was successfully amplified and purified for sequencing; DBH (forward: TGTGTGTCAACTACGTGCACTACTA, reverse: GGCAGGTGCAGACATCCT) on chromosome 25.[13] The DBH primer sequences were taken from the NCBI website; DBH directs the production of the enzyme dopamine beta-hydroxylase, now also known as dopamine beta-monooxygenase, that converts dopamine to norepinephrine.[13]

Primers were amplified in a 25 µl reaction containing 12.5 µl GeneMate Taq 2X Mastermix (concentrations are proprietary information), 2 µl BSA 1X (0.1 mg/ml), 0.5 µl

forward primer, 0.5 µl reverse primer, 2 µl genomic DNA, and 7.5 µl $H_2O$ in that order. PCR reaction conditions for DBH involved an initial denaturing step of 94°C for 3 minutes, followed by 40 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 45 seconds, with a final elongation of 72°C for 3 minutes.[12] All PCR amplifications were carried out on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany).

Purification of PCR amplifications was done following the QIAquick PCR Purification protocol (QIAGEN Corporation, Valencia, CA). 100 µL Buffer PB was added to 20 µL of PCR sample, placed in a QIAquick spin column and collection tube, and centrifuged for 60 seconds at 13,000 rpm. The flow-through was discarded and the spin column was placed back in the collection tube. 0.75 mL Buffer PE was added to the QIAquick column and centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded and the column was placed back in the collection tube, and centrifuged again for 1 minute at 13,000 rpm. The QIAquick column was placed in a 1.5 ml microcentrifuge tube and 30 µl of Buffer EB was added to the center of the QIAquick membrane and incubated at room temperature for 1 minute before centrifuging for 1 minute at 13,000 rpm. The purified DNA was stored at 4°C.

**Sequencing**

Following PCR purification, samples were prepared for sequencing using the Beckman Coulter Sequencing Reaction protocol (Beckman Coulter, Inc., Brea, CA). A 10 µL reaction consisting of 5 µL $H_2O$, 5 µL DNA template, 2 µL forward primer, and 8 µL Beckman Coulter DTCS Quick Start Master Mix (concentrations are proprietary

information) in that order. The reaction was then duplicated substituting 2 µL of reverse primer in place of the forward primer. PCR conditions for the sequencing reaction involved 30 cycles of 96°C for 20 seconds, 50°C for 20 seconds, and 60°C for 4 minutes. The sequencing reaction was cleaned using the Beckman Coulter Ethanol Precipitation protocol (Beckman Coulter, Inc., Brea, CA). Each sample was placed into a 1.5 µL microcentrifuge tube with fresh Stop Solution/Glycogen mixture: 2 µL of 3M Sodium Acetate (pH 5.2), 2 µL 100 mM $Na_2$-EDTA (pH 8), and 1 µL glycogen per sample. 60 µL cold 95% ethanol/$dH_2O$ was added to each sample and vortexed for 10 seconds before immediately centrifuging at 13.4 rpm for 20 minutes. The supernatant was carefully removed, leaving behind a pellet. The pellet was rinsed two times with cold 200 µL 70% ethanol/$dH_2O$; for each rinse the samples were centrifuged immediately at 13.4 rpm for 4 minutes. After each centrifugation the supernatant was carefully removed with a pipette. . After the final rinse, samples were air dried for 10-15 minutes and resuspended in 20 µL of Beckman Coulter Sample Loading Solution (concentrations proprietary information).

All samples were analyzed with a Beckman Coulter CEQ 8000 Genetic Analysis System (Beckman Coulter, Inc., Brea, CA). Sequences were identified by the CEQ 8000 Software and verified by visual inspection before further analysis was performed (Figure I.6).

**Analysis**

Before sequence alignment could occur, reverse sequences were entered into the "Reverse Complement" program[17] to acquire a reverse complement strand. Then each

sequence, the forward strand and reverse complement strand, were entered into EMBOSS: Needle[18] for alignment. This allowed for any disagreements in the original nucleotide sequence to be corrected. Finally, a complete forward strand could be constructed with the corrected information generated from EMBOSS: Needle[18] to create a composite sequence; in the event that a sample only yielded a reverse strand, the reverse complement was found and then the complement strand was found, giving us the required forward strand. Each composite sequence was entered into Clustal Omega[19] for multiple sequence alignment (MSA), allowing areas of similarity to be identified. A Phylogenetic Tree was generated from the Clustal Omega[19] MSA to determine if there was any noteworthy relationship between PPID positive equines.

**RESULTS**

The control group consisted of 13 Quarter horses, 4 Paints, 2 Gypsy Vanners, 3 Arabians, 1 Hackney, 1 Irish Sport Horse, 3 Thoroughbreds, 2 Mustangs, 1 Tennessee Walking Horse, 1 Hackney/Arabian, 1 Irish Sport Horse/Quarter Horse/Thoroughbred, 1 Quarter Horse/Paint, and 1 Thoroughbred/Paint/Quarter Horse of varying ages and sexes. The PPID positive/suspected group consisted of 7 Quarter horses, 1 Appendix Quarter horse, and 1 Arabian; of these, 3 Quarter horses were diagnosed as PPID positive. Five of the six suspected horses display classic equine hypertrichosis and all demonstrated delayed or incomplete shedding. Four of the six suspected horses displayed wasting of the topline and pot belly, and one of the six exhibited extreme polydipsia and polyuria.

Using the NCBI recommended primer pair for DBH, approximately 450 – 550 base pairs were successfully sequenced from each horse, of the 32,248,600 base pair gene. Of the sequences obtained, 26 were both the Forward and Reverse strands, 14 were only the Forward strand, and 6 were only the Reverse strand.

This loci was compared to the fully sequenced DBH gene (NCBI Ref. Seq.: NW_001867396.1) using a Clustal Omega[19] (MSA) to locate its position relative to the entire gene, and highlight areas of similarity between individual horses (Table I.2), allowing any specific regions that have been highly conserved to be found. This locus appears to be highly variable among individual horses, with no apparent specificity among breeds, and with many inserts or possible deletions. The MSA was able to identify 10 areas of similarity among every horse sampled, which are identified in Table I.2 by the asterisk. These areas of similarity are considered to be "highly conserved" over time, although their purpose is has yet to be classified. An interesting discovery was the 11 base pair short tandem repeat found in almost every horse, identified in Table I.2 as the highlighted region, however, what is even more exciting is its complete absence or inversion in other horses. Of the 45 horses sequenced, 4 had a deletion of this short tandem repeat, 3 had an inversion, and 1 displayed a completely different sequence. This repeat did not correspond to the PPID group, or show breed specificity, and at this time its function is unknown.

Using Clustal Omega[19] a Phylogenetic Tree was constructed to determine the relationship within the PPID group (Figure I.7). No relationship was determined to be

present based off the results of this tree; the PPID group was distributed throughout the Phylogenetic Tree, with no noteworthy clustering. Individual breeds were dispersed with no apparent pattern as well, within both the PPID group and the control group.

## DISCUSSION

Ideally this study would have discovered an indication of the genetic nature of PPID; however, the complex nature of this disorder makes targeted research difficult. A large, broad-scaled study will be required to ascertain what genes are the sources of PPID, and what environmental factors trigger them to damage the dopaminergic neurons. In addition, the ability to perform familial studies could provide vital information into the heredity of PPID, which would allow owners and veterinarians the ability to take preventative measures before symptoms appear.

### Limitations

Preferably I would have had a 50/50 ratio of asymptomatic horses and PPID positive horses, with a minimum of 50 asymptomatic and 50 PPID positive horses; unfortunately, due to the death of many potential PPID positive horses and the lack of coordination between veterinarians and owners, samples were not easily obtained and the sample size remained small. Additionally, the limited funding of the university Biology department restricted the scope of this research project, disallowing the addition of multiple gene sites that could have further enhanced the understanding of the mechanism of this multisymptom disorder.

Our molecular biology lab utilizes Sanger Sequencing, which is ideal when working with small fragments of DNA, however, the gene for DBH alone is 32,248,600 base pairs, which is much larger than what Sanger Sequencing is designed for. Because of this, smaller fragment sizes were used, making isolation of any specific mutated regions difficult. Ideally, Next Generation Sequencing should be used to interrogate the millions of base pairs that make up the loci of interest in PPID. Furthermore, this research would greatly benefit from the addition of the gene sites related to equine dopamine receptors; these sites were originally included at the beginning of this study, though the lack of adequate amplification and/or purification caused them to be removed at this time.

**Future research**

While the exact cause of PPID is still unknown, it is my opinion that we are closer to finding improved methods of screening for PPID, and understanding the influence genetics and the environment have upon initiating this disorder. I hypothesized that there is a genetic marker, or markers, that could be used to aid in diagnosing PPID or offer early screening. While this particular study offered inconclusive results at one specific locus, roughly 550 base pairs of the 32,248,600 base pair dopamine beta-hydroxylase gene, there is still an extensive amount of research that can be done concerning this gene and other genes related to PPID; DRD1-5, CDNF, and SLC66A3 relate to dopamine receptor genes that have yet to be explored, a more all-encompassing examination of DBH is needed to determine if mutations here increase the vulnerability of dopaminergic neurons to degeneration, and BDNF and SNCA are already used in human studies to

screen for Parkinson's disease. It stands to reason that if researchers are to consider using PPID as a systemic model for PD [7] the same genes used to screen for PD should be used to screen for PPID.

Our understanding of PPID has increased dramatically over the past decade, regarding both the pathophysiology and the clinical signs. Unfortunately, little advancement has been made concerning the early events that lead to the progression of the disease. Like many neurodegenerative conditions, PPID is proving to be a multisystem disorder, affected by both genetics and environmental factors. As our comprehension of this neurodegenerative disorder grows, this knowledge can lead to improved early diagnostic capabilities and preventative measures, not only for horses, but for humans as well.

# CHAPTER 2

# A COMPARISON OF DNA EXTRACTION TECHNIQUES

Throughout the course of this research it has been necessary to identify the DNA extraction technique that would yield the best results. DNA extraction from hair is often difficult, as only the follicle can be used and inhibitors are present in the extraction. It is recommended when extracting from hair that only anagen stage follicles be used to increase genomic DNA yield; however, follicles for this study were not identified as anagen, catagen, or telogen, as this opportunity is not always available. While many DNA extraction protocols exist the following techniques were chosen for their efficiency and ease of use. For each technique 10 samples were tested.

## DNA EXTRACTION

**Gentra PureGene Protocol: Modified** (QIAGEN Corporation, Valencia, CA)

6 hair follicles (2-5 mm in length) were placed in a 1.5 ml microcentrifuge tube with 100 µl Cell Lysis Solution and manually homogenized before the addition of 3 µl proteinase K and incubation at 55°C for 3 hours. An additional 3 µl proteinase K was added, and the sample was incubated at 55°C for 1 hour. After incubation 150 µl Protein Precipitation Solution was added and the sample was vortexed for 10 seconds. The sample was immediately centrifuged for 5 minutes at 13,400 rpm and the supernatant was poured into a clean 1.5 ml microcentrifuge tube; the pellet was discarded. 150 µl 90% isopropanol was added to the sample with 1 µl glycogen, vortexed for 10 seconds, and

centrifuged for 3 minutes at 13,400 rpm. The isopropanol was poured off and the pellet was washed in 150 µl cold 70% EtOH by gently inverting 5 times. The EtOH was carefully removed with a pipet and the tube was then air dried. Once dry 20 µl DNA Hydration Solution was added and the sample was incubated at room temperature for 1 hour.

**QIAgen DNeasy Protocol: Modified** (QIAGEN Corporation, Valencia, CA)

6 hair follicles (2-5 mm in length) were placed in a 1.5 ml microcentrifuge tube and 180 µl Buffer ATL was added. Grinding or disruption of the follicles was not necessary. 20 µl proteinase K was added and vortexed for 10 seconds before incubation at 56°C for 3 hours. After incubation the samples were vortexed for 10 seconds before 200 µl Buffer AL was added. Samples were vortexed for 10 seconds, 200 µl 99% ethanol was added, and samples were vortexed again for 10 seconds. The mixture (including the precipitate) was pipetted into a DNeasy Mini spin column, placed in a 2 ml collection tube, and centrifuged for 1 minute at 8000 rpm. The flow-through and collection tube were discarded, the spin column was placed in a new collection tube, 500 µl Buffer AW1 was pipetted into the spin column and it was centrifuged for 1 minute at 8000 rpm. The flow-through and collection tube were discarded, the spin column was placed in a new collection tube, 500 µl Buffer AW2 was added to the spin column and it was centrifuged for 4 minutes at 13.3 rpm. Again, the flow-through and collection tube was discarded. The spin column was placed in a 1.5 ml microcentrifuge tube and 50 µl Buffer AE was

pipetted directly onto the DNeasy membrane and incubated at room temperature for 1 minute before being centrifuged for 1 minute at 8000 rpm.

**Chelex**

The first Chelex protocol (CP1) followed Gregory P. Boivin, Victor Otaño-Rivera, Amma Boakye, Nadja Grobe, and Mauricio Di Fulvio's research;[16] 6 hair follicles were placed in 200 µl 10% Chelex and boiled for 20 minutes. The samples were then vortexed for 10 seconds and centrifuged for 5 minutes at 13,000 rpm. The samples were removed from the Chelex using a pipet and placed in a clean 1.5 mL microcentrifuge tube.

The second Chelex protocol (CP2) was recommended by Ed Dekloet of Animal Genetics in Tallahassee, FL. 6 hair follicles were placed in 80 µl 10% Chelex with 10 µl proteinase K and 10 µl molecular grade water. The sample was then incubated for 1 hour at 50°C and then boiled for 30 minutes. Immediately after boiling the sample was vortexed for 10 seconds and centrifuged for 1 minute at 13,000 rpm. The sample was removed from the Chelex using a pipet and placed in a clean 1.5 mL microcentrifuge tube.

**PCR AMPLIFICATION**

A set of equine mitochondrial DNA primers (Cytb_F2, 5'GAATCTAACCACGACCAA3'; Cytb_R2, 5'GTGGAGCTAGAGCTTCTT3') were used to test the efficiency of the genomic DNA extractions. Primers were selected for their known ease of amplification. Primers were amplified in a 25 µl reaction containing

4.5 µl $H_2O$, 2 µl genomic DNA, 2 µl forward primer, 2 µl reverse primer, 2 µl BSA 1X (0.1 mg/ml), and 12.5 µl GeneMate Taq 2X Mastermix (concentrations proprietary information) in that order. PCR reaction conditions involved an initial denaturing step of 94°C for 3 minutes, followed by 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 45 seconds, with a final elongation of 72°C for 3 minutes.

**RESULTS AND DISCUSSION**

When DNA was extracted using the PureGene and DNeasy extraction methods higher yields were obtained verses both Chelex protocols (Figure II.1). After viewing on a 1% agarose gel following electrophoresis, it was clear that while the PureGene and DNeasy protocols yielded high levels of genomic DNA there was also a significant amount of fragmentation and shearing, indicating the presence of inhibitors. The Chelex 1 and Chelex 2 protocols produced low levels of genomic DNA with minimal fragmentation or shearing, indicating a cleaner product (Figure II.2). The PCR product was analyzed on a 2% agarose gel and little difference was observed between extraction methods and amplification, and low levels of shearing were observed with each method.

In addition to the recovery of DNA, several factors must be considered when selecting a DNA extraction method. Table II.1 displays the cost per sample, the minimum processing time, and the volume of DNA recovered for each extraction method. Both Chelex methods were the least expensive methods, required little additional labor, and extractions were obtained quickly. The Chelex methods were problematic when being boiled, as even the boil-proof microcentrifuge tubes had a tendency to pop open, allowing

for contamination. These methods also had the highest level of variability among extractions, making it difficult to replicate the higher yields given with other techniques; this variability could be due to the popping of the tubes during boiling. The PureGene method was the most expensive approach, required the most processing time, and resulted in the lowest sample volume, though the DNA yield between samples had little variability and were reproducible in additional samples. The DNeasy method required longer lyse time and additional manipulations when compared to the Chelex methods, however, produced the highest yields of DNA and gave reasonably reproducible results in additional samples. Should the problem with the tubes be resolved in the Chelex methods, extraction with either Chelex method would be the most efficient and cost-effective way of performing DNA extractions with hair. Because this lab is not set up to boil multiple samples easily and effectively, the DNeasy method of DNA extraction was chosen as the best method for this particular study and lab.

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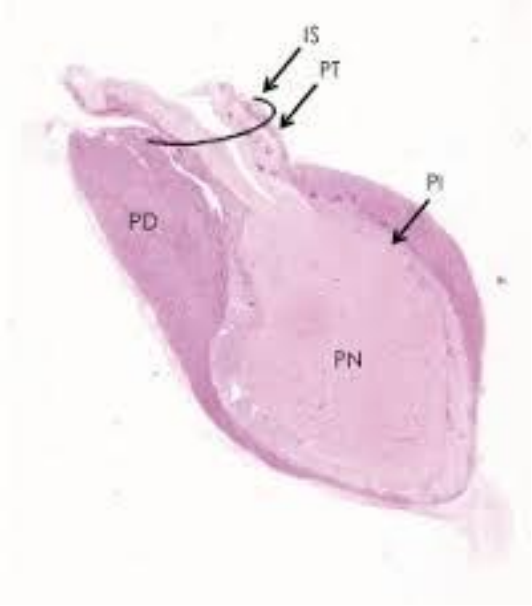


**Figure I.1. Median sagittal section of an equine pituitary gland. Haematoxylin and eosin stained section of a pituitary gland from a normal horse. The pars intermedia (PI) is a narrow band of endocrine tissue located between the pars distalis (PD) and pars nervosa (PN). The pars tuberalis (PT) surrounds the infundibular stem (IS). (2)**

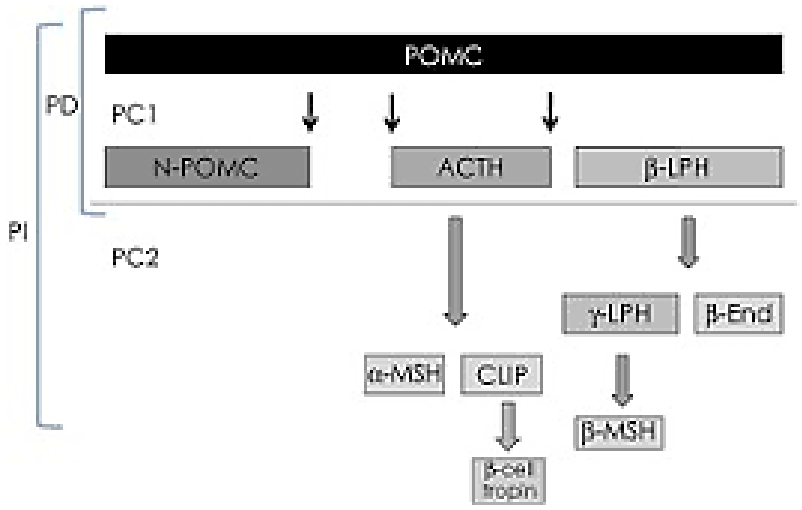


**Figure I.2. Processing of the pro-opiomelanocortin (POMC) peptide in the pars intermedia by prohormone convertase (PC) 1 and 2 cleaves the POMC precursor to yield adrenocorticotrophin (ACTH) and β-lipotrophin (β-LPH). PC2 acts on the products of PC1 cleavage to yield smaller peptides. γ-LPH, γ-lipotrophin; β-END, β-endorphin; α-MSH, α-melanocyte stimulating hormone; CLIP, corticotropin-like intermediate lobe peptide; β-MSH, β-melanocyte stimulating hormone. (2)**

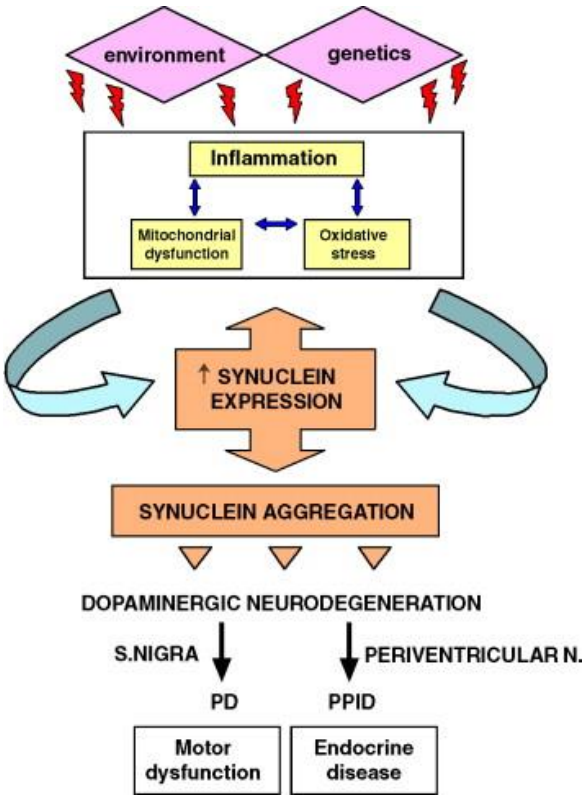


**Figure I.3. A simplified model for the pathogenesis of dopaminergic neurodegeneration. Similar events may occur in Parkinson's disease and equine pituitary pars intermedia dysfunction. In this model, the common central event in both diseases is synuclein accumulation leading to dopaminergic neurotoxicity. Synuclein expression and accumulation is influenced by endogenous and exogenous factors including events that induce oxidative stress. In the case of humans with Parkinson's disease, the most severely affected neurons are those of the substantia nigra. In the horse with pituitary pars intermedia dysfunction, the periventricular neurons are most severely affected. (7)**

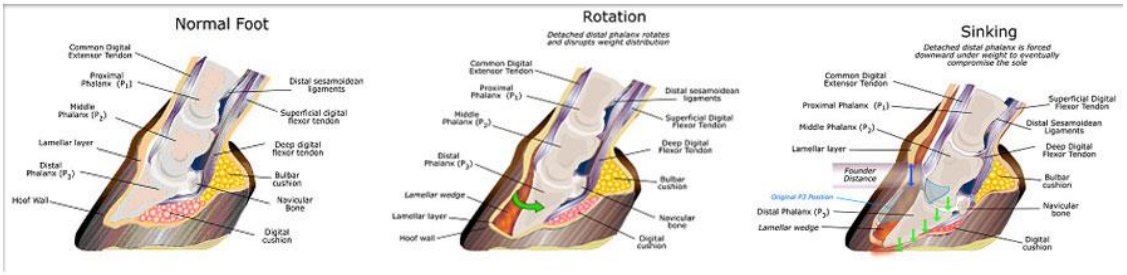


**Figure I.4. The normal equine hoof, traditional laminitis, and the more severe situation, sinking of the coffin bone in the laminitic hoof. Illustrations and format-JamesOrsini, Dvm ACVS. Equine Laminitis in McGraw-Hill yearbook of science and technology. 2008, 114-118**

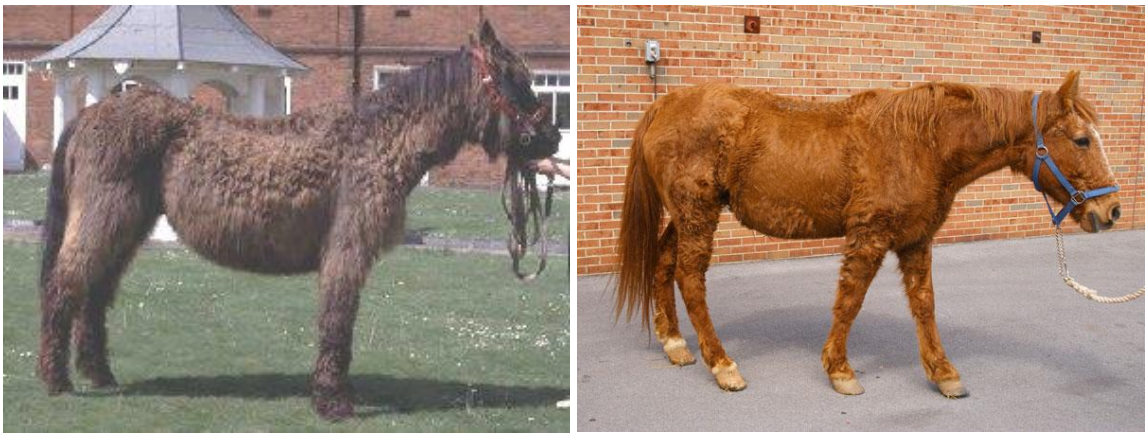

**Figure I.5. Advanced PPID. The horse on the right displays hypertrichosis, skeletal muscle atrophy, and wasted topline and pot belly. The horse on the right displays hypertrichosis, wasting topline, and abnormal hoof growth.**

**(Left) Dr. David Ramey, Encino, CA. <http://www.doctorramey.com/equine-cushings-pars-pituitary-intermedia-disorder-ppid/ >**

**(Right) Equine Endocrinology Group, <http://sites.tufts.edu/equineendogroup/advanced-pituitary-pars-intermedia-dysfunction/>**

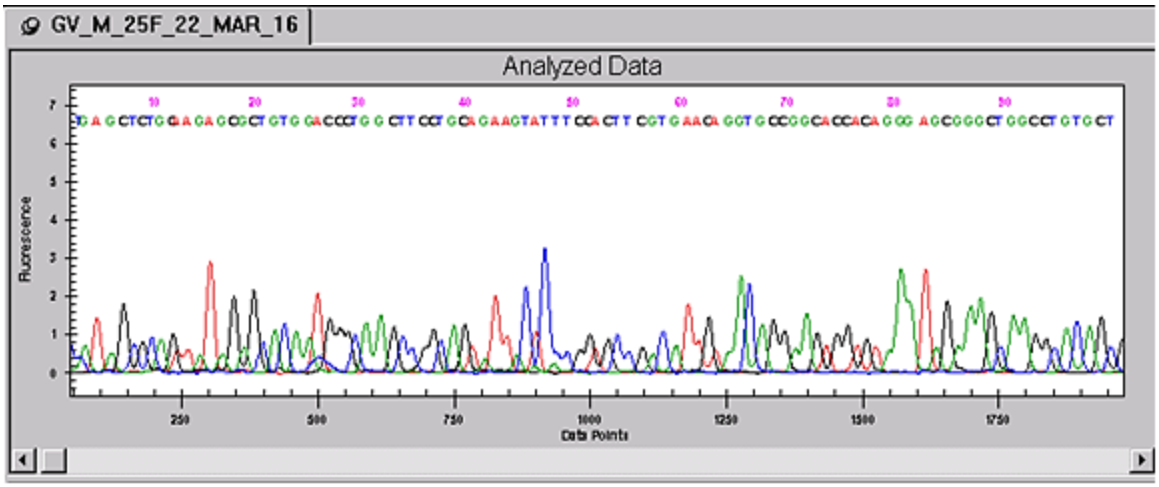


**Figure I.6. Section of an analyzed sequence of DBH in a Gypsy Vanner mare using the Beckman Coulter CEQ 8000 Genetic Analysis System Software.**

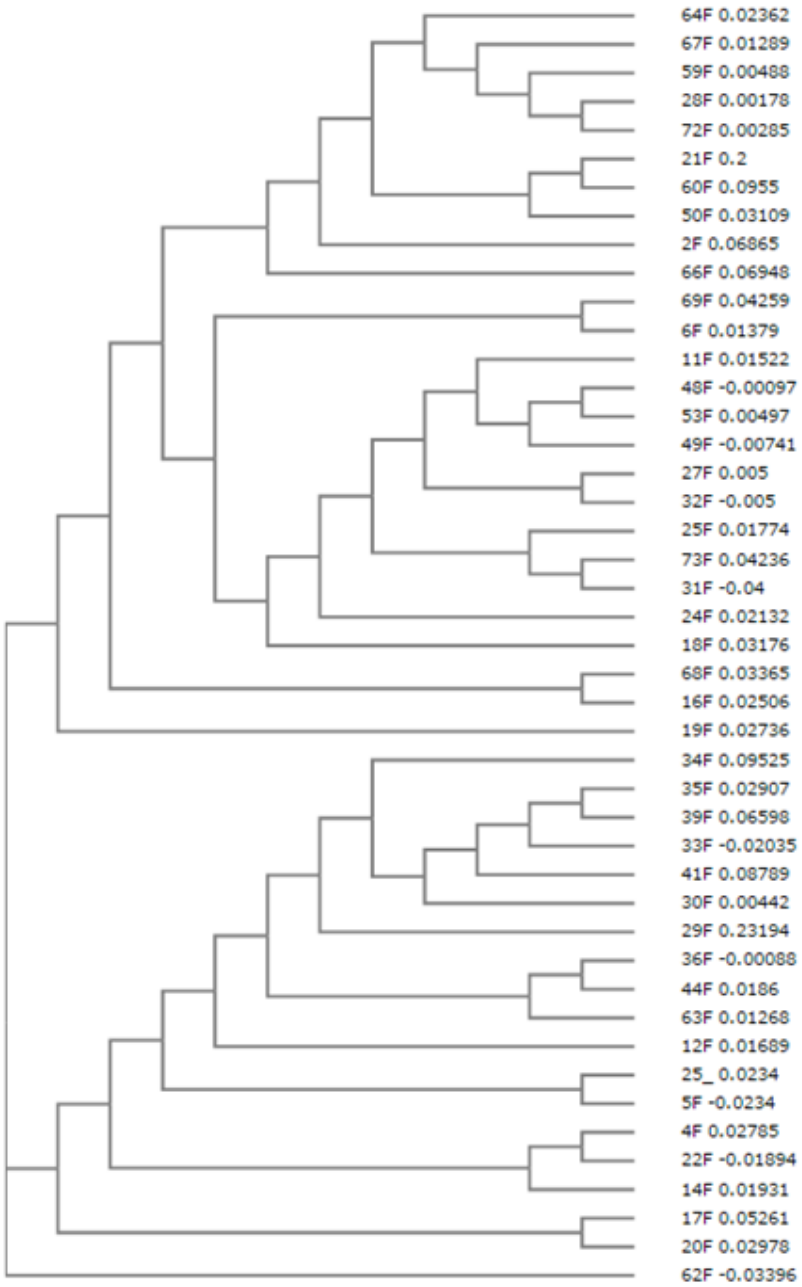


**Figure I.7. Phylogenetic Tree created using Clustal Omega. Each number denotes a specific sample/animal. Numbers 34, 63, 64, 66, 67, 68, 69, 72, and 73 are the PPID positive/suspect animals.**

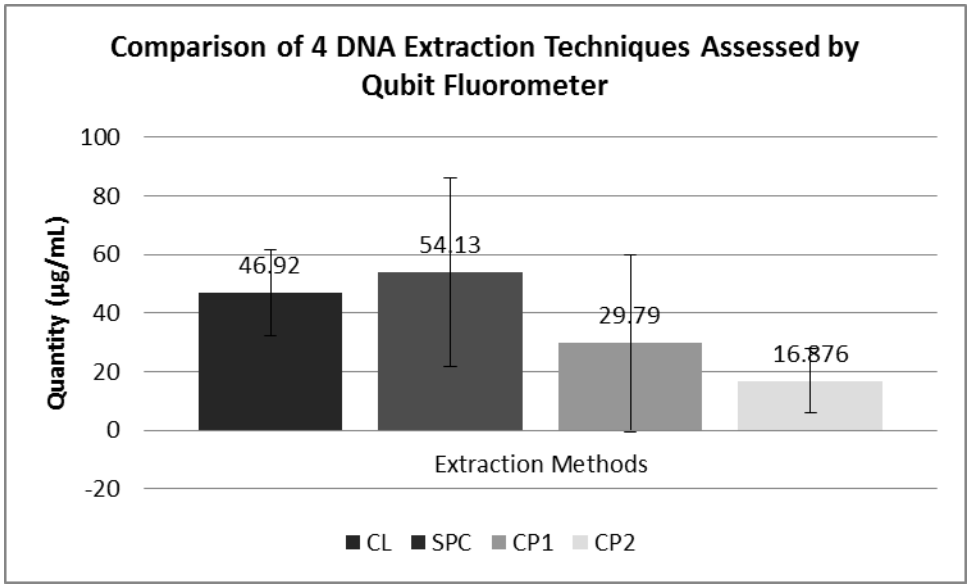


**Figure II.1. Mean levels of genomic DNA as assessed by the Qubit Fluorometer comparing four DNA extraction methods. Error bars indicate standard deviations for replicate extractions. The Gentra PureGene (CL) and QIagen DNeasy (SPC) extraction methods produced the highest levels of genomic DNA from horse hair.**

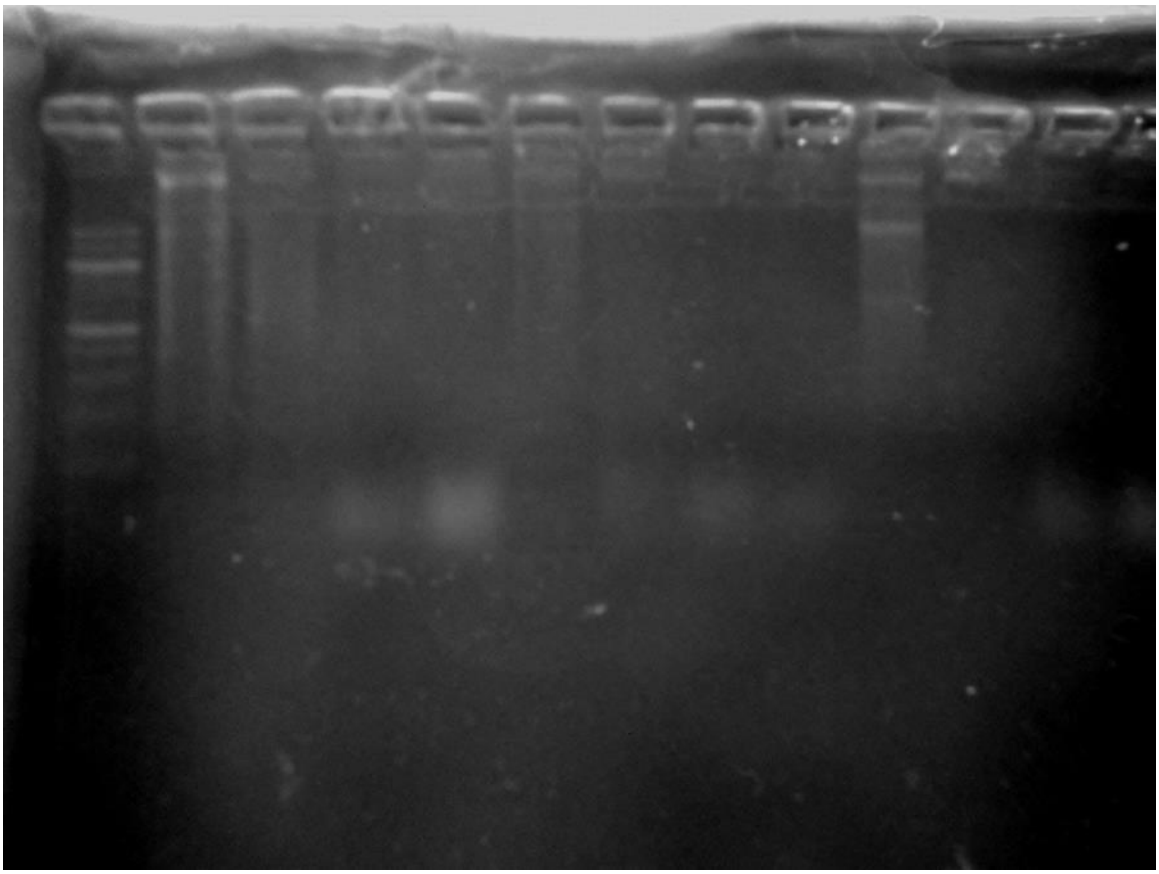

**Figure II.2. Comparison of four DNA extraction techniques displayed on a 1% agarose gel. Wells 2, 6, and 10 contain extractions using the DNeasy protocol. Wells 3, 7, and 11 contain extractions using the PureGene protocol. Wells 4, 8, and 12 contain extractions using the Chelex 1 protocol. Wells 5, 9, and 13 contain extractions using the Chelex 2 protocol.**

| | Hair Samples | | | | | | |
|---|---|---|---|---|---|---|---|
| **#** | **Horse Name** | **Breed/Age/Sex** | **Positive** | **Suspected** | **Negative** | **PCR** | **SEQUENCED** |
| 1 | Cheyenne | Hackney/4/M | | | Negative | | |
| 2 | Rose | Hackney/20/M | | | Negative | * | F/R |
| 3 | Dancer | Hackney/9/St | | | Negative | | |
| 4 | Devon | GV/7/G | | | Negative | * | F/R |
| 5 | Kensey | GV/12/M | | | Negative | * | F |
| 6 | Mia | TB/7/M | | | Negative | * | F/R |
| 7 | Sensei | TB/25/G | | | Negative | | |
| 8 | Sajak | TB/12/G | | | Negative | * | |
| 9 | Colony Times | TB/14/M | | | Negative | * | R |
| 10 | Kola | ARAB/20+/G | | | Negative | * | F |
| 11 | Sage | TB/20+/G | | | Negative | * | F/R |
| 12 | Leo | TB/12/G | | | Negative | * | F/R |
| 13 | Cushing's Pony | Pony/15/G | Positive | | | | |
| 14 | Misty | Pony/NA/M | | | Negative | * | F/R |
| 15 | Holly | TW/9/M | | | Negative | | |
| 16 | Pheonix | TW/15/G | | | Negative | * | F/R |
| 17 | Indian | Mustang/15/G | | | Negative | * | F/R |
| 18 | Kazooie | Kiger Mustang/18/G | | | Negative | * | F/R |
| 19 | Callie | APHA/17/M | | | Negative | * | F |
| 20 | Jim | Pinto/12/G | | | Negative | * | F/R |
| 21 | Spook | Paint/24/G | | | Negative | * | F/R |
| 22 | Apache | Paint/14/G | | | Negative | * | F |
| 23 | Scotch | Paint/18/M | | | Negative | | |
| 24 | Scout | QH/Paint/12/G | | | Negative | * | F/R |
| 25 | Guiness | ISH/20/G | | | Negative | * | F/R |
| 26 | Dish | TB/Paint/12/G | | | Negative | | |
| 27 | Strider | TB/QH/ISH/5G | | | Negative | * | F/R |
| 28 | CJ | TB/Paint/QH/8/G | | | Negative | * | R |
| 29 | Montana | Hack/ARAB/4/G | | | Negative | * | F/R |
| 30 | Katie | ARAB/?/M | | | Negative | | F |
| 31 | Cuesta | ARAB/27/M | | | Negative | * | F |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 32 | Spirit | ARAB/20/G | | | Negative | * | F |
| 33 | Boss | QH/8/G | | | Negative | * | F |
| 34 | Bessta | ApQH/12/M | | Suspected | | * | F/R |
| 35 | No Name | QH/24/G | | | Negative | * | F |
| 36 | Gypsy | QH/?/M | | | Negative | * | F |
| 37 | Tulia | QH/21/M | | | Negative | | |
| 38 | Tater | QH/5/G | | | Negative | * | |
| 39 | Kinetic | QH/4/G | | | Negative | * | F |
| 40 | Belle | QH/9/M | | | Negative | | |
| 41 | Hollywood | QH/12/G | | | Negative | * | |
| 42 | Molly | QH/13/M | | | Negative | | |
| 43 | Pat | QH/30/G | | | Negative | * | |
| 44 | Cooper | QH/8/G | | | Negative | * | F/R |
| 45 | No Name | QH/29/G | | | Negative | | |
| 46 | No Name | QH/20/G | | | Negative | | |
| 47 | No Name | QH/10/G | | | Negative | | |
| 48 | Artisan | QH/12/G | | | Negative | * | F/R |
| 49 | Butters | QH/12/M | | | Negative | * | F |
| 50 | Nelly | QH/12/M | | | Negative | * | F/R |
| 51 | No Name | QH/10/M | | | Negative | | |
| 52 | Gypsy | QH/5/M | | | Negative | | |
| 53 | Gidget | QH/3/G | | | Negative | * | F/R |
| 54 | Jack | QH/9/G | | | Negative | | |
| 55 | Dually | QH/9/G | | | Negative | | |
| 56 | Sprocket | QH/4/G | | | Negative | | R |
| 57 | Max | QH/10/G | | | Negative | | |
| 58 | KeeLark | QH/17/G | | Suspected | | | |
| 59 | Major | QH/25/G | | | Negative | * | |
| 60 | Denver | QH/5/G | | | Negative | * | F/R |
| 61 | Missan A Spur | QH/21/G | | | Negative | | |
| 62 | Studly | QH/13/Stallion | | | Negative | * | F |
| 63 | Baba | QH/8/G | | Suspected | | * | F/R |
| 64 | Journey | QH/12/G | | Suspected | | * | R |
| 65 | Dylan | QH/30/G | | Suspected | | * | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 66 | Rugged Dusty | QH/25/G | Positive | | | * | F |
| 67 | Sam | QH(x)/32/G | Positive | | | * | R |
| 68 | Apache | QH/?/G | Positive | | | * | F/R |
| 69 | Classy | QH/24/M | Positive | | | * | F/R |
| 70 | Gus | QH/29/G | | | Negative | * | |
| 71 | Jane | QH/17/M | Positive | | | | |
| 72 | No Name | QH/25/G | | Suspected | | * | F/R |
| 73 | Socks | Arab/31/G | | Suspected | | * | F/R |
| 74 | Sundays Besst | TB/29/G | | | Negative | * | |

**Table I.1. Sample identification, breed, age, sex, and PPID status. Also noted is whether each sample yielded a PCR product and usable sequence.**

**CLUSTAL OMEGA: Multiple Sequence Alignment**

```
64F     ------------------------------------------------------------
67F     ------------------CCC-CTATCCTTGTCGGACCAGAC------------------
59F     -----------------CCCC-CTTTCCTTGTCGGACCAGAC-----------------
28F     -------------------CC-CCTTTCCTGTCGGACCAGAC-----------------
72F     --------------------C-CCCTTCCTGTCGGACCAGAC-----------------
21F     ------------------------------------------------------CTCT
34F     -------------------CT-CGATTGTGAGCGGCACCGTCCATTGCTGCTCTATGAGG
29F     ---------------------------------------------TCTGCAAGAGC
69F     ------------------------------------------------------------
11F     ------------------------------------------------AAGAGC
6F      ------------------------------------------------------------
25F     ---------------------------------------------TCTGCAAGAGC
18F     ---------------------------------------------CTCTGCAAGAGC
73F     ---------------------------------------------TCTGCAAGAGC
48F     ---------------------------------------------TCTGCAAGAGC
49F     ---------------------------------------------TCTGCAAGAGC
24F     ---------------------------------------------TCTGCAAGAGC
53F     ---------------------------------------------TCTGCAAGAGC
27F     ---------------------------------------------TCTGCAAGAGC
31F     ---------------------------------------------TCTGCAAGAGC
32F     ---------------------------------------------TCTGCAAGAGC
35F     -------------------CT-CGATTGTGGCG-GACCGTCTCTTGTGCTCTATAGGATG
33F     ------------------------------------------------------------
39F     ------------------TCT-CGATTGTGG---GGACCGTCCTTGTGCTCTATAGGATG
2F      ------------------------------------------------------------
60F     ------------------------------------------------------------
50F     ---------------------------------------------TCTGCAAGTAGC
66F     ----------------------------------------------TCTGCAGAGC
41F     ----------------------------------------GCGTCTATAGGATGT
36F     ----------------------------------------------TCTGCTAGTAGC
44F     ----------------------------------------------TCTGCAAGTAGC
30F     ------------------------------------------------------------
19F     ----------------------------------------------CCTCTGCAGAGC
68F     ----------------------------------------------TCTGCAAGTAGC
17F     -----------------------------------------TCTGCAAGTAGCG
20F     ----------------------------------------------TCTGCAAGAGC
25:     TGTGCGTCAACTACGTGCA---CTACTACCCAC-AGACACAGCTTGAGCTCTGCAAGAGC
5F      ----------------------------------------------TCTGCAAGAGC
16F     -GCAAGAGCGCTGTGGACCCTGGCTTCCTGCAG-AAGTATTTCCACTATCGTGCAAGAGC
4F      ------------------------------------------------------------
22F     ---------------------------------------------TCTGCAAGAGCG
14F     ------------------------------------------------------AGAGCG
63F     ---------------------------------------------TCTGCAAGTAGC
12F     ------------------------------------------------------AGC
62F     ------------------------------------------------------------
```

```
64F     ------------------------------------------------------------
67F     -CCACCC--------------------------GTGCTACACCTACCCCTCGGGTAGT--
59F     -CCA----------------------------CCCGTCTACCCTCCCCTCGGGTCGT--
28F     -CCA----------------------------CCCGTCTACCCTCCCCTCGGGTCGT--
72F     -CCA----------------------------CCCGTCTACCCTCCCCTCGGGTCGT--
21F     GCAGAGCGCGTGGTCCGGCTCCCTGCAGAAGTATTTCCCTATCGTGAACAGGTGCCGG--
34F     CATGTAC--------------TTGCTACCCATGCCTACATCGCTGAAACAGGTGCCGGCA
29F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
69F     ------------------------------------------------------------
11F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
6F      ---------------------------------------------TGAACAGGTGCCGG--
25F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
18F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
73F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
48F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
49F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
24F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
53F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
27F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
31F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
32F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
35F     TCTTGCTACCCTAGCCACTCCTAAAC-A------CGGTGACCGAGCACCCACTGCGGT--
33F     ------------------------------------------------------------
39F     TCTGCACC-----TGCCATGCTCAAG-C------ACGGTGCCGAGCCGCCACTGCGGT--
2F      ------------------------------------------------------------
60F     -------------------------AAGTATTTCCCTTCGTGAACACGGTGCCGGCA--
50F     GCTGTGGACCCTGGCTTC---CTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
66F     GCTGTGGACCCTGGCTT----TCTGCATGAATATTCCACTATCGTGAACAGGTGCCGG--
41F     GCTTGCTACCCTGCCTCTCGCTAAAA-C------ACGGTGACCGAGCACCACTAGGGT--
36F     GCAGTGGACC-CTGCGCTTTCTGCATGAAGTATCTCCACTATCGTGAACAGGTGCCGG--
44F     GCTGTGGACC-CTGCGCTTTCTGCATGAAGTATTTCCACTATCGTGAACAGGTGCCGG--
30F     ------------------------------------------------------------
19F     GCTAGTGACCCTGGC-----TTCCTGCAAAGTATTTCCCTATCGTGAACAGGTGCCGG--
68F     GCTGTGGACCCTGGCTTTCTGCATGAAGTATGTTTCCACTATCGTGAACAGGTGCCGG--
17F     CATGTGGACCCTGCGCTTTC--TGCAGTAAGATTTCCACTATCGTGAACAGGTGCCGG--
20F     GCTGTGGACCCTGGCTTCCT--GCAGAAGTA--TTTCCACTTCGTGAACAGGTGCCGG--
25:     GCTGTGGACCCTGGCTTCCT--GCAGAAGTA--TTTCCACTTCGTGAACAGGTGCCGG--
5F      GCTGTGGACCCTGGCTTCCT--GCAGAAGTA--TTTCCACTTCGTGAACAGGTGCCGG--
16F     GCTGTGGACCCTGGCTTCCT--GCAG-AAGTATTTCCACTATCGTGAACAGGTGCCGG--
4F      --TGTGGACCCTGGCTTCCT--GCAGAAGTATGTTCCACTATCGTGAACAGGTGCCGG--
22F     CTAGTGGACCCTGGCTTCCT--GCAGAAG-TATTTCCACTATCGTGAACAGGTGCCGG--
14F     CTAGTGGACCCTGGCTTCCT--GCAGAAG-TATTTCCACTATCGTGAACAGGTGCCGG--
63F     GCTGTGGACCCTGGCTTCCT--GCAGAAG-TATTTCCACTATCGTGAACAGGTGCCGG--
12F     GCTGTGGACCCTGGCTTCCT--GCAGAAG-TATTTCCACTATCGTGAACAGGTGCCGG--
62F     --TGTGGACCCTGGCTTCCT--GCAGAAG-TATTTCCACTATCGTGAACAGGTGCCGG--


64F     ----------------------------------------------------CGACGG
67F     --CCCACACGACTGGGGAGGGCGTCTGAACCATCCCAGAGGCCTCACCGGGGTCCGACGG
59F     --CCCACACGACTGGGGAGGGCGTCTGAACCATCCCAGAGGCCTCACCGGGGTCCGACGG
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28F     --CCCACACGACTGGGGAGGGCGTCTGAACCATCCCAGAGGCCTCACCGGGGTCCGACGG
72F     --CCCACACGACTGGGGAGGGCGTCTGAACCATCCCAGAGGCCTCACCGGGGTCCGACGG
21F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
34F     CCACTAGGGTAGCGGAGCTGGCCTGTGCTC----TCTGACCCCGGGCATGG--CAGGGAC
29F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC--T-CTGATCTGCAAGAG-CGCTGTGGACC
69F     ------------------------------------------------------------
11F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
6F      -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
25F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
18F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCAGGCA-TG--GCAGGGC
73F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
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49F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
24F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
53F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCAGGCA-TG--GCAGGGC
27F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCAGGCA-TG--GCAGGGC
31F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCAGGCA-TG--GCAGGGC
32F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCAGGCA-TG--GCAGGGC
35F     -AGACGGAGTCGTGGCGCTGTCGCGTCCTA--C-TGACGCCCGTGGCA-TGGCAGGGACG
33F     ---------------------------------------------TGGCAGCGGACTG
39F     -AGACGGAGTCGTGGCGCTGCTCGCGCCAC--T-GACGCACCGTGGCATGGCAGCGGACG
2F      ------------------------------------------------------------
60F     -CCACACGGGATGCGGGCTGGCCTGTGCTC--T-TCTGACCCCGGGCA-TGGCACGGGCG
50F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
66F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TGG-GCAGGGC
41F     -AGACGGAGTCGTGGCGCTGCTCGCTCCTA----CTGACCCCGTGGCA-TGGCAGCGGAC
36F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
44F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
30F     ---------------------------CTC----TCTGCACCCGGGCT-AG--GCAG---
19F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
68F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
17F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
20F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCAGGCA-TG--GCAGGGC
25:     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
5F      -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
16F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
4F      -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
22F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
14F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
63F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCGGGCA-TG--GCAGGGC
12F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCAGGCA-TG--GCAGGGC
62F     -CACCACAGGGAGCGGGCTGGCCTGTGCTC----TCTGACCCCAGGCA-TG--GCAGGGC


64F     GGACGGACCGAAGGATTCGTAGCCCGTCTAGGACCGGG---ACCGAGGTGCGGTCACTAC
67F     GGACGGACCGAAG-----GATTGTGCGTTGGAACCGGG---ACCGAGGTGCGGTCACTAC
59F     GGACGGACCGAAG-----GATTGTGCGTCGGAACCGGG---ACCGAGGTGCGGTCACTAC
28F     GGACGGACCGAAG-----GATTGTGCGTTGGAACCGGG---ACCGAGGTGCGGTCACTAC
72F     GGACGGACCGAAG-----GATTGTGCGTCGGAACCGGG---ACCGAGGTGCGGTCACTAC
21F     GGCCCCAACACAGAA-ACCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
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34F     GGCCCCAACAC-AGAAACCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
29F     CTGGCTTCCTGCAGAAGTATTTCCACTATCGTGAACAGGTGCCGGCACCACAGGGAGCGG
69F     ----------------------------------------------------AAGCCAA
11F     GGCCCCAACACAGAA-ACCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
6F      GGCCCCAACACAGAA-ACCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
25F     GGCCCCAACACAGAA-ACCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
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35F     GCCCCATACACATGAAACCCCTGGGCCCTGGAAGGCAGTGGGTGGCAGTGAGGAAGCCAA
33F     GCACCATACACTGAGACCCTCTGGGCCCTGGAAGGCAGTGGGTGGCAGTGAGGAAGCCAA
39F     GCACCATACAC-TGAGACCCCTGGGCCCTGGAAGGCAGTGGGTGGCAGTGAGGAAGCCAA
2F      --------------AAACCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
60F     GCCCCAACACACGAAACCCCTGGGTCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
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41F     GGCCCCAACACAGAAACCCCTGGGCCCTGGGAAGGCAGTGGGTGGCAGTGAGGAAGCCAA
36F     GGCCCCAACACAGAAA-CCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
44F     GGCCCCAACACAGAAA-CCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
30F     --GGCGGCCCACCAGA-ACCCCTGGGCCCTGGGAGCAGTGGGTGGCAGTGAGGAAGCCAA
19F     GGCCCCAACACAGAAA-CCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
68F     GGCCCCAACACAGAAA-CCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
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20F     GGCCCCAACACAGAAA-CCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
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22F     GGCCCCAACACAGAAA-CCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
14F     GGCCCCAACACAGAAA-CCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
63F     GGCCCCAACACAGAAA-CCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA
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62F     GGCCCCAACACAGAAA-CCCCTGGGCCCTGGGAGGCAGTGGGTGGCAGTGAGGAAGCCAA


64F     C-G--ATTACCCGAAGCAGAGAGAGAGAACACG-----GTGGTACCC-ACCCGACCCTTA
67F     C-G--ATTACCCGAAGCAGAGAGAGAGAACACG-----GTGGTACCC-ACCCGACCCTTA
59F     C-G--ATTACCCGAAGCAGAGAGAGAGAACACG-----GTGGTACCC-ACCCGACCCTTA
28F     C-G--ATTACCCGAAGCAGAGAGAGAGAACACG-----GTGGTACCC-ACCCGACCCTTA
72F     C-G--ATTACCCGAAGCAGAGAGAGAGAACACG-----GTGGTACCC-ACCCGACCCTTA
21F     GGATTCAAGAGTCTCGTCCCACTCGCGCCTACATCGCAGACATGGA--------------
34F     GCTA-AGTTCTCAGAGCAGGGTGAGCGCGGATGTAGCGTCTGTACC--------------
29F     -----GCTGGCCTGTGCTCTCTGAGGCAGTGGGTGGCAGTGAGGAAGCCAAGCTAAGTTC
69F     GCTA-AGTTCTCAGAGCAGGGTGAGCGCGGATGTAGCGTCTGTACCTCCATGCCCCCTTG
```

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11F     GCTA-AGTTCTCAGAGCAGGGTGAGCGCGGATGTA------------------------
6F      GCTA-AGTTCTCAGAGCAGGGTGAGCGCGGATGTAGCGTCTGTACCTCCATGCCCCCTTG
25F     GCTA-AGTTCTCAGAGCAGGGTGA---------------------------------
18F     GCTA-AGTTCTCAGAGCAGGGTGAGCGCGGATGTAGCGTCTGTACCTCCATGCCCCCTTG
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62F     GCTA-AGTTCTCAGAGCAGGGTGAGCGCGGATGTAGCGTCTGTACCTCCATGCCCCCTTG


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72F     C---------------------CGAGGAGGTCCCGA---CCGACCTCCCCCGAAGAGTCC
21F     --GGTACGGGGGAAC-------CTCACGGACACCGTCCGCAGTGATTACCTAGGGGTGTG
34F     ----------------------------------------TCCATGGTCCTGCTGAG---
29F     TCAGAGCAGGGTGAGGCGTCA-CTAATGGATCCCCA---CACTCATGTCCTGCTGAG---
69F     GAGTGCCTGTGGCAGGCGTCA-CTAATGGATCCCCA---CACTCATGTCCTGCTGAG---
11F     --------------------------GCGATCCCCA---CACTCATGTCCTGCTGAG---
6F      GAGTGCCTGTGGCAGGCGTCA-CTAATGGATCCCCA---CACTCATGTCCTGCTGAG---
25F     --------------GGCGTCA-CTAATGGATCCCCA---CACTCATGTCCTGCTGAG---
```

```
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49F     GAGTGCCTGTGGCAGGCGTCA-CTAATGGATCCCCA---CACTCATGTCCTGCTGAG---
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2F      GAGTGCCTGTGGCAGGCGTCA-CTAATGGATCCCCA---CACTCATGTCCTGCTGAG---
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16F     GAGTGCCTGTGGCAGGCGTCAGCTAATGGATCCCCA---CACTCATGTCCTGCTGAG---
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22F     GAGTGCCTGTGGCAGGCGTCA-CTAATGGATCCCCA---CACTCATGTCCTGCTGAG---
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62F     GAGTGCCTGTGGCAGGCGTCA-CTAATGGATCCCCA---CACTCATGTCCTGCTGAG---
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64F     GGTTCAGGTCGAGTGGTCCTGTACTCACACCCCTAGGTAAT------------------
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59F     GGTTCAGGTCGAGTCGTCCTGTACTCACACCCCTAGGTAATCACTGCGGACGGTGTCCGT
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21F     AGTACAGGACGACTCGACCTGAACCGGACTCTTCGGGGGAGGTCGGTCGGGACCTCCTCG
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73F     -------CTGGACTTGGCCTGAGAAGCCCCCTCCAGCCAGCCCTGGAGGAGCCATTCCCA
48F     -------CTGGACTTGGCCTGAGAAGCCCCCTCCAGCCAGCCCTGGAGGAGCCATTCCCA
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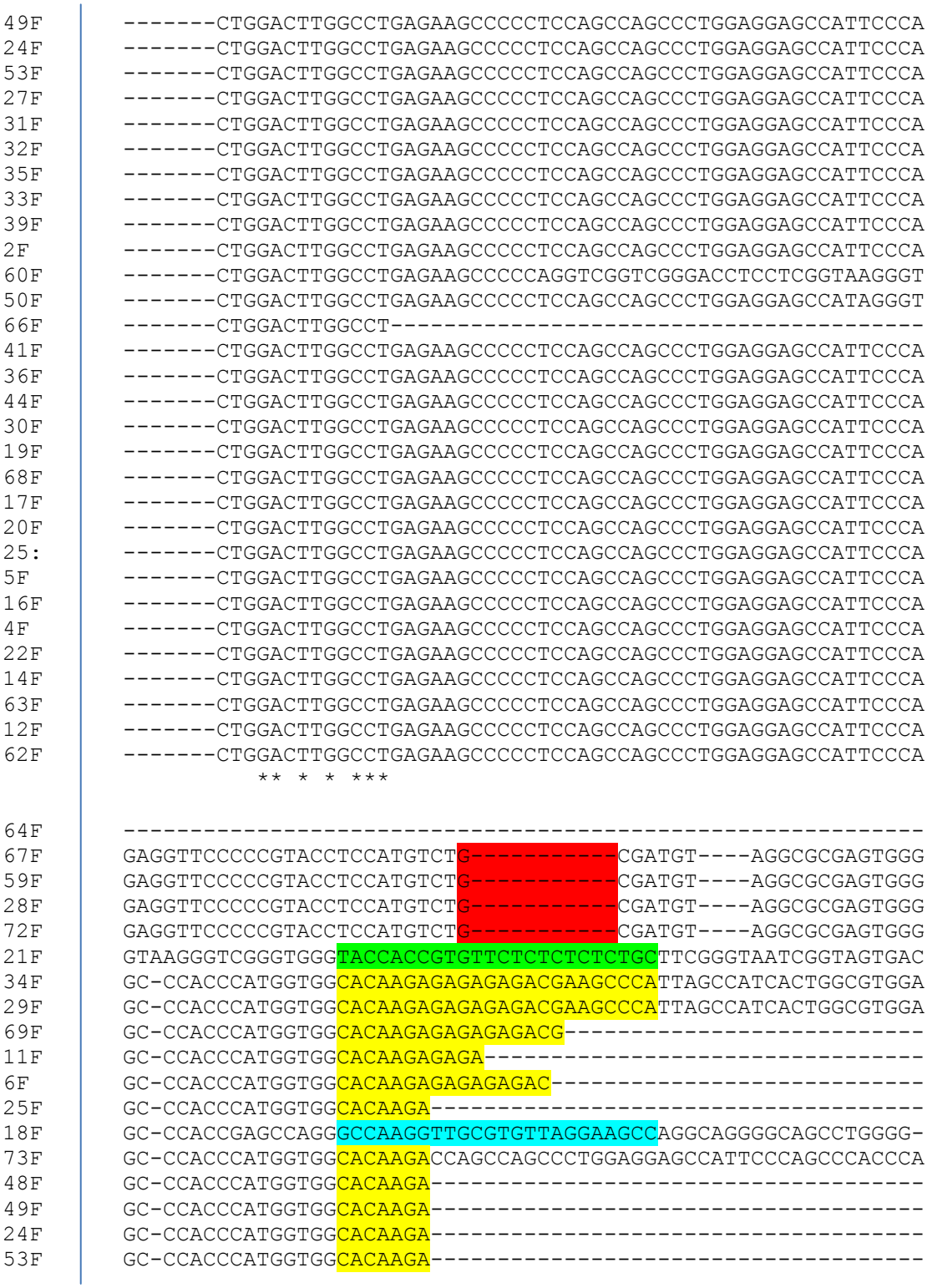
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53F -------CTGGACTTGGCCTGAGAAGCCCCCTCCAGCCAGCCCTGGAGGAGCCATTCCCA
27F -------CTGGACTTGGCCTGAGAAGCCCCCTCCAGCCAGCCCTGGAGGAGCCATTCCCA
31F -------CTGGACTTGGCCTGAGAAGCCCCCTCCAGCCAGCCCTGGAGGAGCCATTCCCA
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39F -------CTGGACTTGGCCTGAGAAGCCCCCTCCAGCCAGCCCTGGAGGAGCCATTCCCA
2F -------CTGGACTTGGCCTGAGAAGCCCCCTCCAGCCAGCCCTGGAGGAGCCATTCCCA
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63F -------CTGGACTTGGCCTGAGAAGCCCCCTCCAGCCAGCCCTGGAGGAGCCATTCCCA
12F -------CTGGACTTGGCCTGAGAAGCCCCCTCCAGCCAGCCCTGGAGGAGCCATTCCCA
62F -------CTGGACTTGGCCTGAGAAGCCCCCTCCAGCCAGCCCTGGAGGAGCCATTCCCA
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72F GAGGTTCCCCCGTACCTCCATGTCTG----------CGATGT----AGGCGCGAGTGGG
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11F GC-CCACCCATGGTGGCACAAGAGAGA----------
6F GC-CCACCCATGGTGGCACAAGAGAGAGAGAC----------
25F GC-CCACCCATGGTGGCACAAGA----------
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73F GC-CCACCCATGGTGGCACAAGACCAGCCAGCCCTGGAGGAGCCATTCCCAGCCCACCCA
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39F GC-CCACCCATGGTGGCACAAGAGAGAGAGACGAAGCCCATTAGCCATCACTGGCGTGGA
2F GC-CCACCCATGGTGGCACAAGAGAGAGAGACGAAGCCCAAATCGGTAG-------TGAC
60F CG--------GGTGGGTACCACCGTGTTCTCTCTCTCTGCTTCGGGTAATCGGTAGTGAC
50F CG-GGTGGGTACCACCGTGTTCTCT-------CTCTCTGCTTCGGGTAATCGGTAGTGAC
66F ----------
41F GC-CCACCCATGGTGGCACAAGAGAGAGAGACGAAGCCCATTAGCCATCACTGGCGTGGA
36F GC-CCACCCATGGTGGCACAAGAGAGAGAGACGAAGCCCATTAGCCATCACTGGCGTGGA
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68F GC-CCACCCATGGTGGCACAAGAGAGAGAGACGAAGCCCATTAGCCATCACTGGCGTGGA
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12F GC-CCACCCATGGTGGCACAAGAGAGAGAGACGAAGCCCATTAGCCATCACTGGCGTGGA
62F GC-CCACCCATGGTGGCACAAGAGAGAGAGACGAAGCCCATTAGCCATCACTGGCGTGGA
64F ----------
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59F ACGAG---ACTCTTGAATCGAACCGAAGGAGTGACGGTGGGTGACGGAGGGTCCCGGGTC
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72F ACGAG---ACTCTTGAATCGAACCGAAGGAGTGACGGTGGGTGACGGAGGGTCCCGGGTC
21F ----CGCACCTCGGTCCCGGTTCCGACGCACAATCCTTCGGTCCGTCCCCGTCGG-----
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29F GCCAG-------GGCCAAGGCTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
69F ----------AAGCCCATTAGCCAT-----CACTGGCGTGGAGCCAG
11F ----------GAGACGAAGCCCATTAGCCAT-----CACTGGCGTGGAGCCAG
6F ----------GAAGCCCATTAGCCAT-----CACTGGCGTGGAGCCAG
25F ----------GAGAGAGACGAAGCCCATTAGCCAT-----CACTGGCGTGGAGCCAG
18F -----CCACTCCGGAGAGGGATGGT--TCAGACGCCCT-----CCCCAG----------
73F TGGTGGCACAAGAGAGAGAGACGAAGCCCATTAGCCAT-----CACTGGCGTGGAGCCAG
48F ----------GAGAGAGACGAAGCCCATTAGCCAT-----CACTGGCGTGGAGCCAG
49F ----------GAGAGAGACGAAGCCCATTAGCCAT-----CACTGGCGTGGAGCCAG
24F ----------GAGAGAGACGAAGCCCATTAGCCAT-----CACTGGCGTGGAGCCAG
53F ----------GAGAGAGACGAAGCCCATTAGCCAT-----CACTGGCGTGGAGCCAG
27F ----------GAGAGAGACGAAGCCCATTAGCCAT-----CACTGGCGTGGAGCCAG
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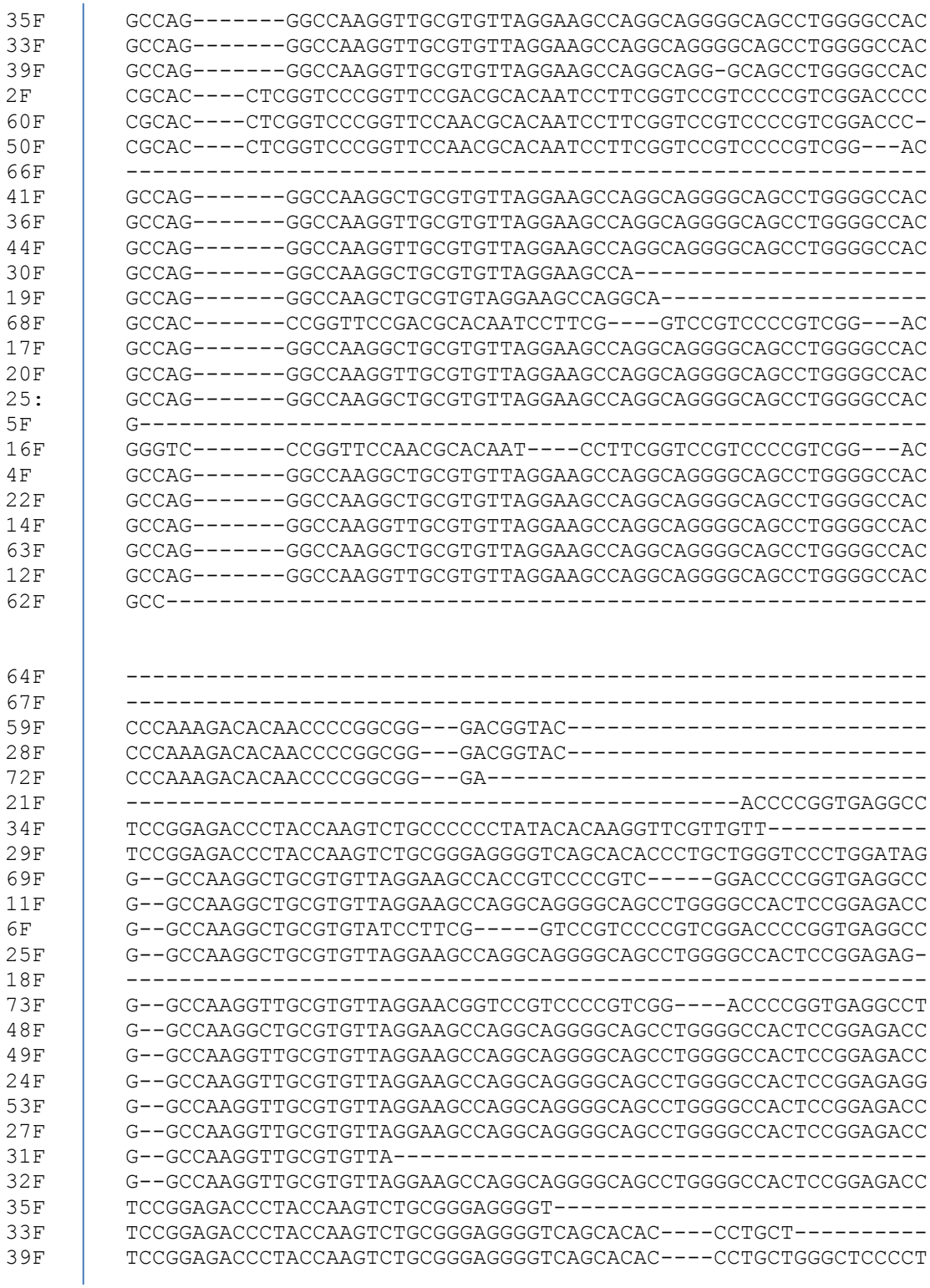
32F ----------GAGAGAGACGAAGCCCATTAGCCAT-----CACTGGCGTGGAGCCAG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39F GCCAG-------GGCCAAGGTTGCGTGTTAGGAAGCCAGGCAGG-GCAGCCTGGGGCCAC
2F CGCAC----CTCGGTCCCGGTTCCGACGCACAATCCTTCGGTCCGTCCCCGTCGGACCCC
60F CGCAC----CTCGGTCCCGGTTCCAACGCACAATCCTTCGGTCCGTCCCCGTCGGACCC-
50F CGCAC----CTCGGTCCCGGTTCCAACGCACAATCCTTCGGTCCGTCCCCGTCGG---AC
66F ----------
41F GCCAG-------GGCCAAGGCTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
36F GCCAG-------GGCCAAGGTTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
44F GCCAG-------GGCCAAGGTTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
30F GCCAG-------GGCCAAGGCTGCGTGTTAGGAAGCCA----------
19F GCCAG-------GGCCAAGCTGCGTGTAGGAAGCCAGGCA----------
68F GCCAC-------CCGGTTCCGACGCACAATCCTTCG----GTCCGTCCCCGTCGG---AC
17F GCCAG-------GGCCAAGGCTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
20F GCCAG-------GGCCAAGGTTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
25: GCCAG-------GGCCAAGGCTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
5F G----------
16F GGGTC-------CCGGTTCCAACGCACAAT----CCTTCGGTCCGTCCCCGTCGG---AC
4F GCCAG-------GGCCAAGGCTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
22F GCCAG-------GGCCAAGGCTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
14F GCCAG-------GGCCAAGGTTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
63F GCCAG-------GGCCAAGGCTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
12F GCCAG-------GGCCAAGGTTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCAC
62F GCC----------
64F ----------
67F ----------
59F CCCAAAGACACAACCCCGGCGG---GACGGTAC----------
28F CCCAAAGACACAACCCCGGCGG---GACGGTAC----------
72F CCCAAAGACACAACCCCGGCGG---GA----------
21F ----------ACCCCGGTGAGGCC
34F TCCGGAGACCCTACCAAGTCTGCCCCCCTATACACAAGGTTCGTTGTT----------
29F TCCGGAGACCCTACCAAGTCTGCGGGAGGGGTCAGCACACCCTGCTGGGTCCCTGGATAG
69F G--GCCAAGGCTGCGTGTTAGGAAGCCACCGTCCCCGTC-----GGACCCCGGTGAGGCC
11F G--GCCAAGGCTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCACTCCGGAGACC
6F G--GCCAAGGCTGCGTGTATCCTTCG-----GTCCGTCCCCGTCGGACCCCGGTGAGGCC
25F G--GCCAAGGCTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCACTCCGGAGAG-
18F ----------
73F G--GCCAAGGTTGCGTGTTAGGAACGGTCCGTCCCCGTCGG----ACCCCGGTGAGGCCT
48F G--GCCAAGGCTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCACTCCGGAGACC
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53F G--GCCAAGGTTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCACTCCGGAGACC
27F G--GCCAAGGTTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCACTCCGGAGACC
31F G--GCCAAGGTTGCGTGTTA----------
32F G--GCCAAGGTTGCGTGTTAGGAAGCCAGGCAGGGGCAGCCTGGGGCCACTCCGGAGACC
35F TCCGGAGACCCTACCAAGTCTGCGGGAGGGGT----------
33F TCCGGAGACCCTACCAAGTCTGCGGGAGGGGTCAGCACAC----CCTGCT----------
39F TCCGGAGACCCTACCAAGTCTGCGGGAGGGGTCAGCACAC----CCTGCTGGGCTCCCCT

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2F      G---GTGAGGCCTCTGGGATGG--------------------------------------
60F     --CGGTGAGGCCTCTGG------------------------------------------
50F     CCCGGTGAGGCCTCTGGGA----------------------------------------
66F     ----------------------------------------------------------
41F     TCCGGAGACCCTACCAAGTCTGCCCCCTTCACCAAGTTCG----TTGTTCGGTCGCT---
36F     TCCGGAGACCCTACCAAGTCTGCGGGAGGGGTCAGCACAC----CCTGCTGGGCT-----
44F     TCCGGAGACCCTACCAAGTCTGCGGGAGGGGTCAGCACAC----CCTGATCCCGAGGGGA
30F     ----------------------------------------------------------
19F     ----------------------------------------------------------
68F     CCCGGTGAGGCCTCGTGGGATGGTTCAGACG----------------------------
17F     TCCGGAGAGGGATGGTTCAGACGCCCTCCCCAGTCGTGTG----GGACAA-------CCC
20F     TCCGGAGAGGGATGGTTCAGACGCCCTCCCC-------AG----TCGTGTGGGACGACCC
25:     TCCGGAGACCCTACCAAGTCTGCGGGAGGGGTCAGCACAC----CCTGCTGGGCTCCCCT
5F      ----------------------------------------------------------
16F     CCCGGTGAGGCCTCT--------------------------------------------
4F      TCCGGAGAGGCTGCGTGTTAGGAAGCCAGGCAGGGGCAGC----CTGGGGCCACTCCGGA
22F     TCCGGAGA----------------------------------------------------
14F     TCCCAGGGAGGGATATGCGGGTGGTGCGGTAACAACG----------------------
63F     TCCGGAGACCCTACCAAGTCTGCGGGAGGGGTCAGCACAC----CCTGCTACCCCCAGGG
12F     TCCGGAGACCCTACCAAGTCTGCGGGAGGGGTCAGCACAG----GGACGACCCAAGGGAG
62F     ----------------------------------------------------------


64F     ----------------------------------------------------------
67F     ----------------------------------------------------------
59F     ----------GGGCCCCAGTCTCTCGTGTCCGGTCGGGCGAGGGACACCACGGCCGTGGA
28F     ----------GGACCCCAGTCTCTCGTGTCCGGTCGGGCGAGGGACACCACGGCCGTGGA
72F     ----------------------------------------------------------
21F     TCTGGGATGGTTCAGACGCCCTCCCCAGTCGTGTGGGACGAC--CCGAGGGGAGGGTAGA
34F     ----------------------------------------------------------
29F     CGGGTGGG---TCTGG--------------------TACCAA------CGGAAA------
69F     TCTGGGATGGTTCAGACGCCCTCCCCAGTCGTGTGGGACAAC--CCACGAGGGAGG----
11F     CTACCAAGTCTGCGGGAGGGG-----AGTCGTGTGGGACGAC--CCGAGGGGAGGGTAGA
6F      TCTGGGATGGTTCAGACGCCCTCCCCAGTCGTGTGGGACGAC--CCGAGGGGAGGGTAGA
25F     ----GGATGGTTCAGACGCCCTCCCCAGTCGTGTGGGACGAC--CCGAGGGGAGGGTAGA
18F     ----------------------------TCGTGTGGGACGAC--CCGAGGGGAGGGTAGA
73F     CTGGGATG-GTTCAGACGCCCTCCCCAGTCGTGTGGGACGAC--CCGAGGGGAGGGTAGA
48F     CTACCAAGTCTGCGGGAGGGGTCAGC-----ACACCCTGCT--------------------
49F     CTACCAAGTCTGCGGGA------------------------------------------
24F     GATGGTTCAGACGCCCTCCCCAGTCG-----TGTGGGACTAC--CCGAGGGGAGGGTAGA
53F     CTACCAAGTCTGCGGGAGGGGTCAGC-----ACACCCTGCTG--GGCTGGGGAGGGTAGA
27F     CTACCAAGTCTGCCCCTCCCCAGTCG-----TGTGGGACGAC--CCGAGGGGAGGGTAGA
31F     ----------------------------------------------------------
32F     CTACCAAGTCTGC----------------------------------------------
35F     ----------------------------------------------------------
33F     ----------------------------------------------------------
39F     CCCATCTGCCCACCCAGACCA--------------------------------------
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60F     -----GATGGTTCAGACGCCCTCCCCAGTCGTGTGGGACGAC--CCGAGGGGAGGGTAGA
50F     -------TGGTTCAGACGCCCTCCCCAGTCGTGTGGGACGAC--CCGAGGGGAGGGTAGA
```

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66F     ------------------------------------------------------------
41F     ------------------------------------------------------------
36F     ------------------------------------------------------------
44F     GGTGTAGCACGGGTGGGTCTGGTCCGACAGGAAGGGG----------------------
30F     ------------------------------------------------------------
19F     ------------------------------------------------------------
68F     -----------------CCCGTCCCCAGTCGTGTGGGACGAC--CCGAGGGGAGGGTAGA
17F     GAGGGGAGGTGTAGACGGGTGGGTCTGGTCC--------GACAAGGAAGGGG--------
20F     GAGGGGAGGG-TAGACGGGTGGGTCTGGTCCGA----CAGGAA----AGGGG--------
25:     CCCATCTGCC-CACCCAGACCAGGCTGTTCCTTTCCCCCTAACCCCCCCAGGTTCAATGG
5F      ------------------------------------------------------------
16F     --GGGATGGT-TCAGACGCCCTCCCCAGTCGTGTGGGACTAC--CCGAGGGGAGGGTAGA
4F      GACCCTACCA-AGTGACGCCCTCCCCAGTCGTGTGGGACGAC--CCGAGGGGAGGGTAGA
22F     ------------------------------------------------------------
14F     ------------------------------------------------------------
63F     TAG-GTATAC-GGTGGGG-------------------------------------------
12F     GGA-GTTCGG-GGTGAAA-------------------------------------------
62F     ------------------------------------------------------------


64F     ------------------------------------------------------------
67F     ------------------------------------------------------------
59F     CAAGTG--------------------------------------------------------
28F     CAAGTGCTTCAC---CTTTATGAAGACGTCCTTCGGTCCCAGGTGTCGCGAGAACGTCTC
72F     ------------------------------------------------------------
21F     CGGGTGGGTCTGGTCCGACAGGAAAGGGG--------------------------------
34F     ------------------------------------------------------------
29F     ------------------------------------------------------------
69F     ------------------------------------------------------------
11F     CGGGTGGGTCTGGTCCGACAAGGAAAGGGG--------------------------------
6F      CGGGTGGGTCTGGTCCGACAAGGAAAGGG---------------------------------
25F     CGGGTGGGTCTGGTCCGACAAGGAAAGGGG--------------------------------
18F     CGGGTGGGTCTGGTCCGACAAGGAAGGGG---------------------------------
73F     CGGGTGGGTCTGGTCCGACAGGAAAGGGG--------------------------------
48F     ------------------------------------------------------------
49F     ------------------------------------------------------------
24F     CGGGTGGGTCTGGTCCGACAGGAAAGGGG--------------------------------
53F     CGGGTGGGTCTGGTCCGACAGGAAAGGGGG-------------------------------
27F     CGGGTGGGTCTGGTCCGACAGGAAAGGGG--------------------------------
31F     ------------------------------------------------------------
32F     ------------------------------------------------------------
35F     ------------------------------------------------------------
33F     ------------------------------------------------------------
39F     ------------------------------------------------------------
2F      TGGGTGGGTTGTGCGTCCGAAAGGAAA----------------------------------
60F     CGGGTGGGTCTGGTCCGACAGGAAAGGGG--------------------------------
50F     CGGGTGGGTCTGGTCCGACAGGAAAGGGG--------------------------------
66F     ------------------------------------------------------------
41F     ------------------------------------------------------------
36F     ------------------------------------------------------------
```

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44F     ------------------------------------------------------------
30F     ------------------------------------------------------------
19F     ------------------------------------------------------------
68F     CGGGTGGGTCTGGTCCGCAAGGATAGGGG--------------------------------
17F     ------------------------------------------------------------
20F     ------------------------------------------------------------
25:     CGAGGAAGTCTGCACCTGCCCTCAGGCCTCCGTCCCTGAGCAGTTTGCCACTGTTCCCTG
5F      ------------------------------------------------------------
16F     CGGGTGGGTCTGGCCGACAGGAAAGGGG--------------------------------
4F      CGGGTGGGTCTGGTCCGACAAGGAAAGGGG--------------------------------
22F     ------------------------------------------------------------
14F     ------------------------------------------------------------
63F     ------------------------------------------------------------
12F     ------------------------------------------------------------
62F     ------------------------------------------------------------


64F     ----------------------------------------------------
67F     ----------------------------------------------------
59F     ----------------------------------------------------
28F     GAGTTCGACACAGACACCCATCATCACG------TGCATCATCTGG---------
72F     ----------------------------------------------------
21F     ----------------------------------------------------
34F     ----------------------------------------------------
29F     ----------------------------------------------------
69F     ----------------------------------------------------
11F     ----------------------------------------------------
6F      ----------------------------------------------------
25F     ----------------------------------------------------
18F     ----------------------------------------------------
73F     ----------------------------------------------------
48F     ----------------------------------------------------
49F     ----------------------------------------------------
24F     ----------------------------------------------------
53F     ----------------------------------------------------
27F     ----------------------------------------------------
31F     ----------------------------------------------------
32F     ----------------------------------------------------
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39F     ----------------------------------------------------
2F      ----------------------------------------------------
60F     ----------------------------------------------------
50F     ----------------------------------------------------
66F     ----------------------------------------------------
41F     ----------------------------------------------------
36F     ----------------------------------------------------
44F     ----------------------------------------------------
30F     ----------------------------------------------------
19F     ----------------------------------------------------
```

| | |
|---|---|
| 68F | ---------- |
| 17F | ---------- |
| 20F | ---------- |
| 25: | GAACTCCTTCAACCGGCAGGTGCTCAGCGCCCTGTACGGCTTCGCCCCTATCTCC |
| 5F | ---------- |
| 16F | ---------- |
| 4F | ---------- |
| 22F | ---------- |
| 14F | ---------- |
| 63F | ---------- |
| 12F | ---------- |
| 62F | ---------- |

**Table I.2. Multiple Sequence Alignment using Clustal Omega. Numbers in the left column represent individual samples/animals. Nucleotides are indicated with either A, T, C, or G in the right column. " — " denotes gaps within the sequences, " * " indicates all sequences have the same nucleotide, fully conserved. The highlighted regions display the short tandem repeat (yellow), its deletion (red), its inversion (green), or the change in sequence (blue).**

| Extraction Method | Processing Time (min) | Volume Recovered | Cost per sample |
|---|---|---|---|
| **DNeasy** | 220 | 50 µL | $2.75 |
| **PureGene** | 345 | 20 µL | $3.75 |
| **Chelex 1** | 25 | 100 µL | $0.51 |
| **Chelex 2** | 101 | 100 µL | $0.51 |

**Table II.1. Comparison of different methods for DNA extraction based on cost, time, and sample volume. The processing time indicates extractions of single samples, the lyse time, and DNA rehydration, if necessary. The additional proteinase K needed for the Chelex 2 extraction method has not been included in the cost per sample.**