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Evaluation of Ranavirus Major Capsid Protein Gene Sequence Mutations in United States East Coast Frog and Tadpole Populations

Erin Marie Tyll

Abstract

Infectious disease is a leading cause of death in ectothermic taxa including amphibians, reptiles, and bony fish. Frog Virus 3 (FV3) *Ranavirus* is a prominent pathogen that infects frog and tadpole populations with lethal consequences, especially for pre-metamorphosed individuals. Wood frogs (*Lithobates sylvaticus*) are particularly susceptible to FV3 infection and mortality events. Conservation of this animal is a critical effort for the maintenance of these ecologically significant animals. Amphibians contribute to the regulation of insect pest levels, energy transfer between aquatic and terrestrial habitats, algae community productivity and function, and vertebrate biomass. Understanding genetic variations of FV3 is crucial to understanding the evolution of this virus and host susceptibility. Diagnostic testing is key for preventing the spread of the virus and reducing the mass mortality events observed in tadpole populations. Populations studied originate from 5 US states, Delaware, Maryland, New Jersey, Pennsylvania, and Virginia. This study explored the validity of current Real Time-PCR diagnostic testing methods and sought out genetic variations that may account for inconsistent diagnostic results brought forth by previous studies. Despite symptom observations in the field and positive fluorescence during diagnostic testing, 416 individuals have been deemed negative as they did not meet the melting temperature (Tm) criteria currently in place. This study presents findings that support the hypothesis that many, if not all, of these individuals are in fact positive for the virus. Due to the prior conflicting diagnostic results the Tm range currently in place may not account for new mutations within a ~530 base pair targeted region of the viral major capsid protein (MCP) gene sequence. Molecular techniques were used to evaluate the amplified DNA of the targeted region of the MCP gene. Traditional PCR and gel electrophoresis results provided evidence of infection in 41 individuals from nine populations. This was followed by sequence analysis of the PCR

product for 22 of those individuals. Sequence results confirmed *Ranavirus* infections in 15 individuals from seven populations. A single base pair mutation was located in six individuals from a New Jersey population.

MONTCLAIR STATE UNIVERSITY

Evaluation of *Ranavirus* Major Capsid Protein Gene Sequence Mutations in United States East Coast Frog and Tadpole Populations

by

Erin Marie Tyll

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Introduction

Infectious disease is a leading cause of death in ectothermic taxa including amphibians, reptiles, and bony fish (Mao et al., 1996a, Jancovich et al., 2001, Chinchar, 2002). Some Ranaviruses are limited to infecting a single class of organisms while others have shown to infect members of multiple classes (Wolf et al., 1968, Moody and Owens, 1994). Ranaviruses are found on all continents except Antarctica (Duffus et al., 2015). *Ranavirus* (Family *Iridoviridae*) is a double-stranded DNA virus with a ~105-140 kbp genome (Essbauer and Ahne, 2001, Williams et al., 2005, Jancovich et al., 2012). The virions are icosahedral and are typically ~150 nm in diameter (Essbauer and Ahne, 2001). It can spread through a population very quickly, killing most, if not all of the individuals present within a few days to a week of infection (Harp and Petranke, 2006, Brunner et al., 2015). Without scientific intervention this virus has the potential to wipe out full populations to entire species (Brunner et al., 2015).

Frog Virus 3 (FV3) is a form of *Ranavirus* capable of infecting frog larvae and adults (Gray et al., 2009, Bienentreu, 2020). Virions may be shed into the environment, spreading to other individuals indirectly through the water or soil which they inhabit (Harp and Petranke, 2006, Hall, 2018). Factors that can contribute to the transmission of the virions through the environment include water chemistry, soil type, ambient temperature, hydroperiod, and UV-B levels (Gray et al., 2009). Direct transmission occurs in amphibian populations through direct contact with an infected individual, predation, and necrophagy (Harp and Petranke, 2006, Gray et al., 2009). Factors that are likely contributors to host susceptibility include natural stressors (development, food limitation, host density, predation, water temperature, and co-infection), anthropogenic stressors (pesticides, fertilizers, nitrogenous waste, heavy metals, acidification,

genetic isolation), and virulence (strain novelty, exposure history, coevolution) (Gray et al., 2009).

Ranavirus infections in amphibians commonly appear as lesions on the body presenting as limb or body swelling, erythema, swollen friable livers, and hemorrhage (Gray et al., 2009, Robert, 2014). Behavioral display of infection may include buoyancy problems, erratic swimming, anorexia, bloody discharge, and lethargy (Gray et al., 2009, Miller et al., 2011). Cause of death in amphibians is organ failure due to chronic cell death (Gray et al., 2009). Amphibian species exhibit different degrees of susceptibility to Ranaviruses. This may be due to their varying co-evolutionary histories with the viral pathogens (Gray et al., 2009). Widespread amphibian population die-offs due to Ranaviruses may be due to an interaction of suppressed and naïve host immunity, anthropogenic stressors, and novel strain introduction (Gray et al., 2009, Savage et al., 2019). Conservation of amphibians is a critical effort for the maintenance of these ecologically significant animals. Amphibians contribute to the regulation of insect pest levels, energy transfer between aquatic and terrestrial habitats, algae community productivity and function, and vertebrate biomass (Daszak et al., 1999, Whiles et al., 2006).

This research has studied the presence of FV3 in US wood frog (*Lithobates sylvaticus*) tadpole populations to monitor the spread and evolution of this pathogen. FV3 exposure lab tests have found that wood frogs appear to have the highest *Ranavirus* susceptibility among 19 North American amphibian species (Hoverman et al., 2011). Populations studied originate from 5 states, Delaware, Maryland, New Jersey, Pennsylvania, and Virginia. Liver and kidney samples were used for DNA extracted as those organs are most expressive of *Ranavirus* infection in individual specimens (Robert et al., 2007, 2011, 2014, Gray et al., 2012). The tissue samples collected from at-risk individuals have been tested for the presence of the virus using Real Time

PCR (RT-PCR) as the standard method for molecular diagnostic analysis (Monsen-Collar, 2013). The molecular diagnostic test, RT-PCR, is used to detect the presence of a targeted fragment (~530 bp long) of the highly conserved *Ranavirus* major capsid protein (MCP) gene (Mao et al. 1996a, Greer et al., 2007, Dolcemascolo, 2014). The viral fragment is amplified and its associated level of fluorescence is reported by the RT-PCR system. Although DNA amplification is often a strong and reliable diagnostic technique, in preliminary studies, the use of this technique has been known to provide amplified results for non-targeted DNA. To ensure the targeted DNA sequence is amplified the melting temperature (Tm) of the amplified DNA is also analyzed and quantified through a melt curve that is generated during each RT-PCR run (Dolcemascolo, 2014). The Tm is the point at which 50% of the amplified DNA is double stranded and 50% is single stranded. This temperature varies depending on the specific sequence of the DNA in question making the melting temperature for the targeted DNA unique.

Currently the accepted Tm for the *Ranavirus* major capsid protein gene sequence is +/- 2°C of the positive control's melting temperature which is typically ~87°C (Smith et al., 2016). As a result of this parameter, amplified results that have a Tm outside the currently accepted range are reported as negative for the virus. An increasing number of false positive fluorescent results with Tm just a few degrees outside of this threshold range have been reported. In addition to being tested in the Monsen-Collar lab some samples were also sent for independent screening for *Ranavirus* by the National Wildlife Health Center (NWHC) (Smith et al., 2016). Four populations deemed negative for the virus by the Monsen-Collar lab were found to be positive by NWHC. Individuals from those populations displayed increased fluorescence but failed to meet the melting curve criteria (Smith et al. 2016). A number of individuals displayed a single melting temperature on the melting curve that was approximately 5°C lower than the positive control's

melting temperature (Smith et al., 2016). Upon further investigation the Monsen lab found that two individuals from population MDMO02 were infected with a genetic variant (Smith et al., 2016). The variant contained a single nucleic acid base pair difference of an adenine base while the positive control contained a guanine base. Adenine and thymine bases have lower melting temperatures when compared to guanine and cytosine bases indicating that mutations such as this one have the potential to affect the melting temperature of the targeted *Ranavirus* DNA (Lando, 2015).

Previous studies may have results outside the temperature range due to possible genetic mutations that may have altered the fragments' melting temperatures. At the time of collection many individuals in the populations in question showed the key symptoms of this virus (erratic swimming, reddening of ventral skin, particularly around the base of the hind limbs and the vent opening, bloated abdomen) (Smith et al. 2016, Monsen-Collar, pers. comm.). Despite symptom observations in the field and positive fluorescence during diagnostic testing, positive fluorescence and symptoms observed prior to this study, 416 individuals have been deemed negative as they did not meet the melting temperature (Tm) criteria currently in place. This study presents findings that support the hypothesis that many, if not all, of these individuals are in fact positive for the virus. Based on the false positive amplifications observed, I suspect the Tm range for the target sequence does not account for new mutations within the targeted region of the major capsid protein gene sequences.

This study set out to identify sequence variants in the ~530 bp fragment of the *Ranavirus* major capsid protein (MCP) gene used for diagnostic testing (Mao et al., 1996b). Currently, the positive controls contain MCP sequences void of any newly developed mutations. Improved understanding of the mutations that are occurring in the capsid protein gene sequence will

facilitate the improvement of the Tm threshold. In addition to improving diagnostic accuracy, genetic relatedness among different *Ranavirus* strains plays an important role in understanding future disease outbreaks. The study of evolutionary relatedness and age of viral strains provides better insight into the virulence of the FV3 virus and possible resistance within the host.

Methods

Genomic DNA Collection

Liver and kidney tissue samples were taken from wood frog larvae which were collected for a previous study (Smith et al. 2016). Thirty wood frog larvae were collected from each population (pond) in 2013 and 2014 at the Gosner stage 27 of metamorphosis (65-130 days post hatching). The tissue samples were euthanized in benzocaine hydrochloride water baths, an American Veterinary Medical Association Panel on Euthanasia approved method (Smith et al. 2016, AVMA 2013, p. 77). This method was also approved for the formerly mentioned study through the Institutional Animal Care and Use Committee (IACUC) reviewed by the National Park Service and Smithsonian Conservation Biology Institute (Smith et al. 2016). Genomic DNA was extracted from the tissue samples using the QIAGEN QIAamp® DNA Mini Kit following the manufacturer's instructions. Populations originated from 5 US states: New Jersey, Delaware, Pennsylvania, Virginia, and Maryland. The extracted total genomic DNA samples were stored in a -80℃ freezer and were thawed at room temperature prior to use.

Polymerase Chain Reaction (PCR)

The positive control was genomic DNA extracted from a wood frog larva that was verified to be infected with FV3. The highly conserved major capsid protein (MCP) region of ~500bp was amplified using forward primer MCP4: 5'-GACTTGGCCACTTATGAC-3' and reverse primer MCP5: 5'-GTCTCTGGAGAAGAAGAA-3' (Mao et al. 1996b). Both MCP4 and MCP5 have been shown to only amplify *Ranavirus* (Mao et al. 1996b). Each PCR reaction (Total 25µL) consisted of deionized water up to 25µL, 1X Promega Colorless GoTaq® Flexi Buffer, 1.5 mM Promega $MgCl_2$, 0.2 mM New England BioLab dNTP Mix (Nucleotide Mix),

0.4 µM of each primer, and 0.05 units/µl Promega GoTaq® Flexi Polymerase. Reaction mixtures were held at 94℃ for 2m30s for initial denaturation, followed by 30 or 35 cycles of denaturation at 94℃ for 30s, annealing at 50℃ for 30s, and extension at 72℃ for 30s. At the completion of 30 or 35 cycles the reactions were held at 72℃ for 10m and then kept at 4℃ until removed from the thermocycler and placed in a -20℃ freezer.

Gel Electrophoresis

Two percent 1X TAE agarose gels were prepared using 2.5µl of SYBR Safe DNA stain (Invitrogen) per 100 ml. Ten microliters of BIONEXUS, Inc. All Purpose Hi-Lo™ DNA Marker 50bp-10,000bp was used as the size standard and 3 µl of 15% ficoll loading dye was added to 5µl PCR product. Gels were run in 1X TAE buffer at 120 volts for approximately 60 minutes. Fluorescence was visualized using the Kodak Gel Logic 200 Imaging System.

Cycle Sequencing Reaction

PCR product concentrations were diluted ten-fold in an attempt to meet the optimal DNA concentration range of 20-50ng/µL (A. Parker, pers. comm.). Each cycle sequencing reaction (total 20µL) consisted of 1.5µL of each sample with nucleic acid concentration ranging from 30.3-778.8 ng/µL, 1.5µL (10µM) forward or reverse primer (MCP4 or MCP5), 0.5µL ThermoFisher Scientific BigDye™ Terminator v3.1 Ready Reaction Mix, 3.75µL 5X ThermoFisher Scientific BigDye™ Sequencing Buffer, and deionized water up to 20µL. Reaction mixtures were held at 96℃ for 1m for initial denaturation, followed by 25 cycles of denaturing at 96℃ for 10s, annealing at 50℃ for 5s, and extension at 60℃ for 2m. At the completion of 25 cycles the reactions were held at 10℃ until removed from the thermocycler.

Cleanup Purification and Loading Plate

Sequencing reactions were purified using ThermoFisher Scientific BigDye™ Sequencing Clean Up Kit. Ten microliters of Molecular Cloning Laboratories (MCLAB) BigDye® Sequencing Clean Up Kit carboxylated magnetic beads were combined with 10µL of the cycle sequencing reaction product and 80% ethanol. The samples were washed using 70% ethanol and were eluted using 40µL of MCLAB Sequencing Clean Up Kit elution buffer. Twenty-five microliters of eluted sequencing reaction was loaded on an Applied Biosystems 3130GA genetic analyzer instrument. Deionized water was used as the negative control and the purified plasmid M13 (-21) Primer, pGEM control DNA was used as the positive control. The DNA was taken up via electrokinetic injection. The voltage for injection was set to the default setting of 12 seconds.

Genetic Analysis

The raw chromatogram sequence results for each individual were compared to a 531 base FV3 MCP reference sequence (Supplementary Figure 1) derived from the positive controls using the National Library of Medicine (NIH) genome database using the Basic Local Alignment Search Tool (BLAST). BLAST was also used to compare the raw chromatogram sequences. SnapGene® Viewer and 4Peaks were used to review peaks and quality values (PHRED scores). Gaps, insertions and mismatches were reviewed and corrected manually if deemed necessary based on peak appearance and quality. The edited DNA sequences were aligned with the 531 base FV3 MCP reference sequence using the SnapGene® 6.0.2 software. The amino acid sequence for FV3 MCP was obtained from the GenBank database and was a 100% match for the control FV3 sequence (Mao et al. 1996b, GenBank AC U36913). The sequence containing thymine at the 372 nucleic acid position of the targeted region of the FV3 MCP was then

converted to an amino acid sequence using the Swiss Institute of Bioinformatics program ExPASy. The FV3 MCP reference amino acid sequence was aligned with the variant amino acid sequence using EMBL's European Bioinformatics Institute Multiple Sequence Alignment (MUSCLE) tool.

DNA Concentration and Purity Analysis

A Thermo Scientific NanoDrop 1000 was used to measure the concentration of DNA in ng/µl. Protein contamination levels were assessed using the 260/280 purity ratio. Non-protein and nucleic acid organic contamination levels were assessed using the 260/230 purity ratio. Two microliters of each sample was measured and the device was blanked using deionized water before each sample was analyzed.

Results

Amplification of Target DNA using Primers MCP4 and MCP5

Three hundred and forty-six samples from 48 populations previously deemed false positives underwent polymerase chain reactions (PCR). Following DNA amplification the products were run through a 2% agarose gel. Forty-one samples from nine populations showed apparent bands near the 500 base pair location of the ladder (Figure 1a-h). Positive (*) and negative (**) controls were used for all gel runs. All results presented originate from PCR runs in which the negative controls showed no signs of contamination. All apparent DNA is detectable through the presence of SYBR Safe. Figure 1a shows DNA that was amplified to a detectable level in the form of fluorescent bands for individual 03 from population DENC12. The positive control failed for the plate in which DNA from DENC12-03 was amplified. The results were deemed valid and the sample was later sequenced. Figures 1b-h all have successful positive and negative controls indicating that the target DNA was amplified to a detectable level when present and there was no detectable contamination. Figure 1b shows DNA that was amplified to a detectable level in the form of fluorescent bands for individuals 368 and 373 from population MDMO02. The fluorescence level for individual 368 appeared to be greater than the band for 373 and the positive control. Figure 1c shows a faint fluorescent band for individual 78 from population NJMO04. Figure 1d shows faint fluorescent bands for individuals 910 and 67 from populations NJSU and DESX respectively in contrast to the positive control fluorescence. Figure 1e shows fluorescent bands for individuals 78, 660-664 from populations DESX02 & MDHO03 respectively. The fluorescence level for individual 660 is the greatest while the other individuals have faint fluorescence when compared to the positive control. Figure 1f shows fluorescent bands for individual 676 from population MDHO03, individuals 689, 691, 707, 694

from population VASH02 and individuals 631, 634, 641-644, 647-649, 651, 657, 658 from population NJWA01. The fluorescence for individuals 647 and 651 appear to be the greatest while faint bands are visible for all other individuals. Figure 1g shows fluorescent bands for individuals 751, 752, 756, 757, 759, 760, 762, 764, 765, 771, 772 from population NJMO04. The fluorescence for individual 760 is the greatest while individuals 752 and 772 have more prominent fluorescent levels when compared to the remaining bands for other individuals. Figure 1h shows fluorescent bands for individual 658 from population DENC07. The fluorescence level is low when compared to the positive control.

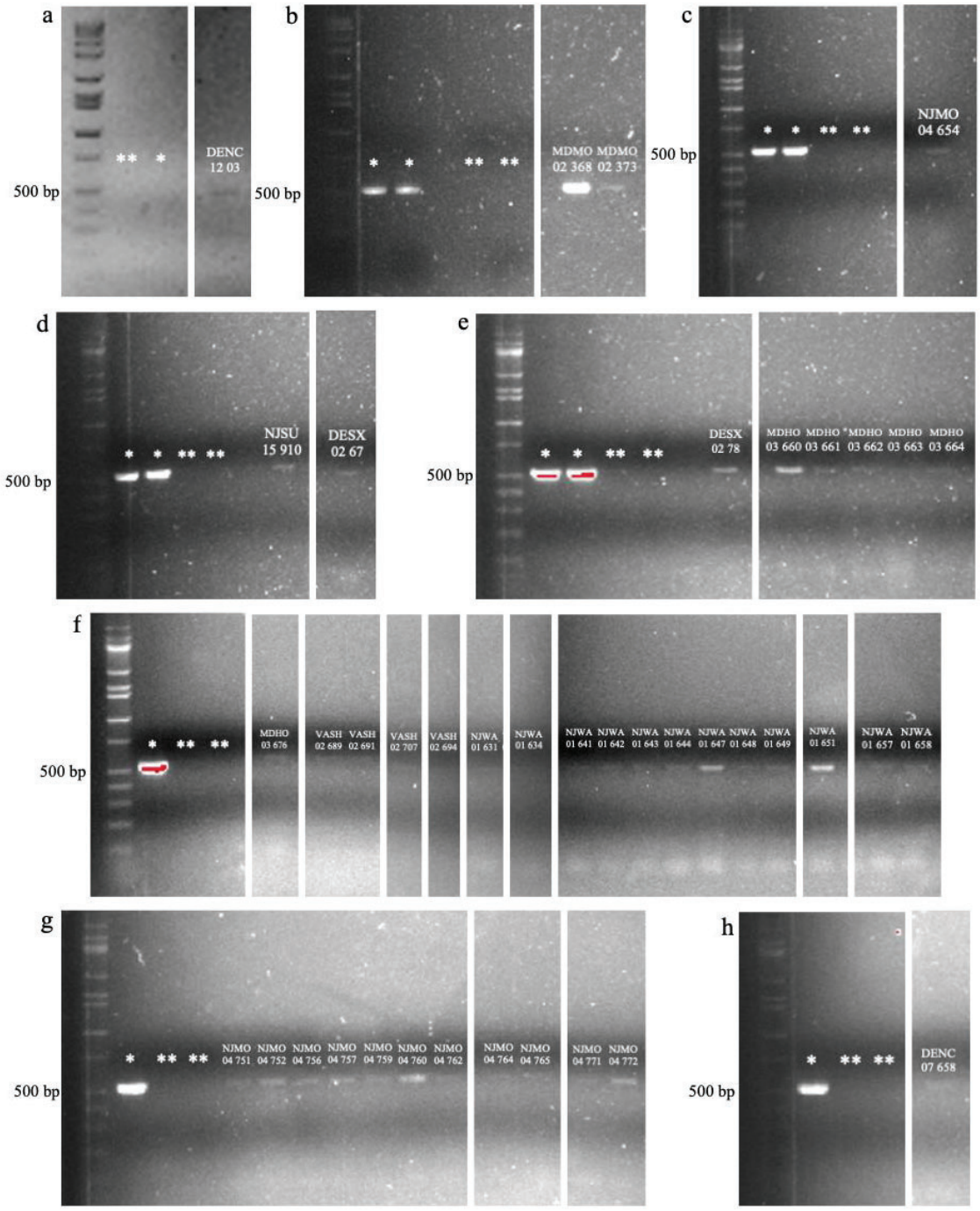

Figure 1: Traditional PCR Product Gel Electrophoresis Results

First lane in all figures is the Hi-Lo Ladder (a-h) Bands present near 500 bp of reference ladder. (a) Individual 03 from population DENC12; (b) individuals 368 & 373 from population MDMO02; (c) individual 654 from population NJMO04; (d) individuals 910 & 67 from populations NJSU15 & DESX02 respectively; (e) individuals 78 & 660-664 from population DESX02 & MDHO03 respectively; (f) individual 676 from population MDHO03; individuals 689, 691, 707, 694 from population VASH02; individuals 631, 634, 641-644, 647-649, 651, 657, 658 from population NJWA01; (g) individuals 751, 752, 756, 757, 759, 760, 762, 764, 765, 771, 772 from population NJMO04; (h) individual 658 from population DENC07.

* Positive Control

** Negative Control

Genetic Analysis

Of the samples that were successfully amplified through traditional PCR, 22 were chosen based on fluorescence and population representation. The sequences were aligned with a sequence for the control FV3 samples (Supplementary Figure 1) using the NIH BLAST alignment function. Based on the sequence alignment results the amplified product from those 15 individuals matched the FV3 MCP targeted gene sequence. The reads for those 15 individuals had percent identity values ranging from 84.73 to 99.79%, with at least one read for each individual >91% (Supplementary Table 3). The E-values for those alignments ranged from 6.00E-71 to zero (Supplementary Table 3). Based on the average quality values (>15) generated by 4Peak software, a total of 21 reads (forward and reverse primer sequences) for 15 individuals were of high enough quality for further analysis (Table 1). Based on quality scores of <20 the first ~20 base pairs following the primer sequence for each read were omitted from further analysis. All apparent peak misreads were manually corrected in the SnapGene Software prior to alignment with the control FV3 sequence. Only the highest quality regions of the reads were used to represent the sequence results for each individual resulting in some sequences that do not span the full target region of ~531 base pairs (Figure 2). Alignment percentages of the query sequences with the reference FV3 MCP ranged from 56.87-100% (Figure 3 & Supplementary Table 4).

Individual	Average Quality of Chromatogram	Total Sequence Length (base pairs)
DENC07 658	R1: 10.9 | R2: 8.9	R1: 407 | R2: 385
DENC12 03	**R1: 33.2 |** R2: 8.8	**R1: 525 |** R2: 147
DESX02 67	**R1: 39.8 |** R2: 14.6	**R1: 506 |** R2: 436
DESX02 78	R1: * R2: *	R1: * R2: *
MDHO03 660	**R1: 42.9** | R2: 5.5	**R1: 493** | R2: 117
MDMO02 368	**R1: 47.3** | R2: 38.7 | R3: 43.6 **| R4: 45.0**	**R1: 497** | R2: 501 | R3: 497 **| R4: 492**
MDMO02 373	**R1: 37.2 | R2: 45.4**	**R1: 539 | R2: 505**
NJMO04 751	**R1: 15.7 | R2: 19.4**	**R1: 409 | R2: 487**
NJMO04 752	**R1: 27.4 | R2: 33.4**	**R1: 409 | R2: 510**
NJMO04 754	**R1: 24.8 | R2: 16.3**	**R1: 458 | R2: 463**
NJMO04 756	**R1: 35.0** | R2: 11.6	**R1: 487** | R2: 484
NJMO04 757	R1: * | **R2: 27.9**	**R2: 508**
NJMO04 760	**R1: 31.1 | R2: 40.0**	**R1: 514 | R2: 504**
NJMO04 772	**R1: 28.7 | R2: 35.9**	**R1: 481 | R2: 508**
NJWA01 632	R1: 13.1 | R2: *	R1: 413
NJWA01 647	**R1: 24. 5 | R2: 34.0**	**R1: 465 | R2: 496**
NJWA01 651	**R1: 39.8** | R2: *	**R1: 494**
NJSU15 910	R1: 16.3 | R2: *	R1: 469
VASH02 689	R1: 10.9 | R2: *	R1: 421
VASH02 691	R1: 12.1 | R2: 11.4	R1: 407 | R2: 455
VASH02 694	R1: 9.0 | R2: 7.1	R1: 0 | R2: 0
VASH02 707	**R1: 24.5** | R2: 11.5	**R1: 474 | R2: 701**

Table 1: 4Peaks analysis of sequences for ten-fold diluted PCR products
Forward primer sequence reads are represented by R1 & R3
Reverse primer sequence reads are represented by R2 & R4
Sequencing runs in bold represent sequences used in multialignment
*No Results

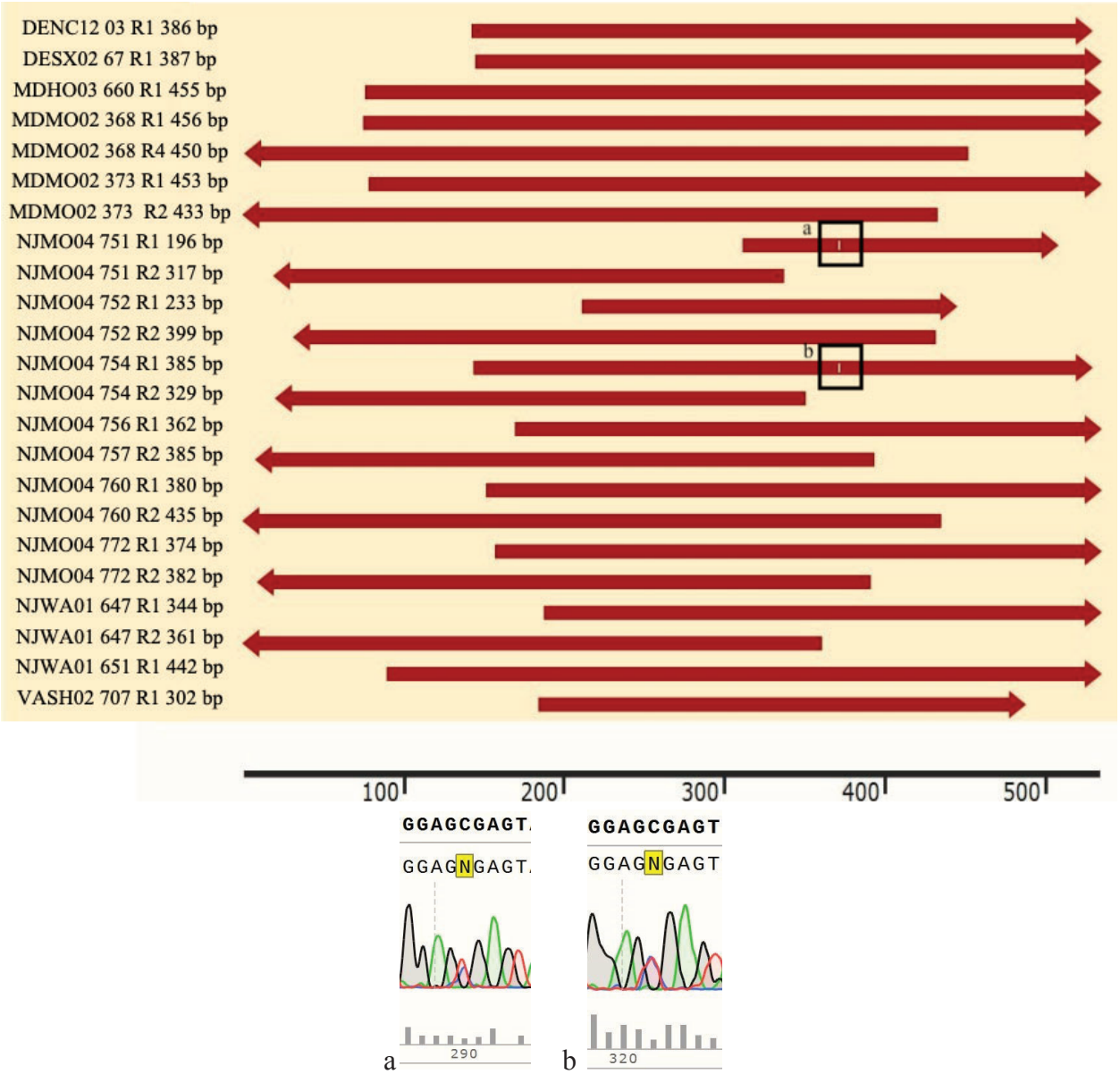

Figure 2: Query sequence alignments with FV3 MCP reference sequence (531bp) including sequence variants

(a) Thymine and cytosine peaks visible at nucleic acid base 372 of read 1 (forward primer) sequence for individual 751 of NJMO04 population. (b) Thymine and cytosine peaks visible at nucleic acid base 372 of read 1 (forward primer) sequence for individual 754 of NJMO04 population.

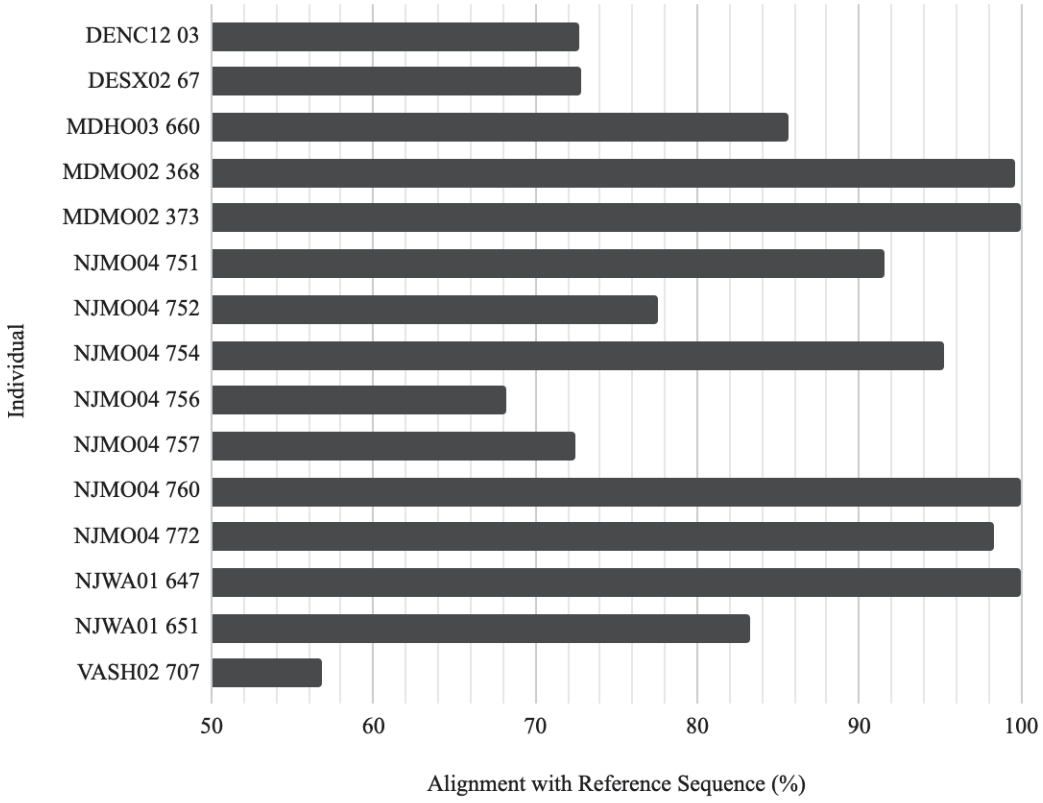

Figure 3: Percent alignment of query sequences with reference FV3 MCP gene target sequence. Percent alignment to the reference sequence of the highest quality sequence results ranges from 56.87-100%.

Measuring Nucleic Acid Concentration

To determine the approximate concentration of nucleic acids in the samples that were used for sequencing, measurements of each sample were recorded using the NanoDrop device. Figure 4 shows the concentration results for the extracted DNA and the final ten-fold diluted PCR products. The full set of measurements including undiluted PCR products can be found in supplementary table 1. All samples noted with * had complete or nearly complete sequencing results that matched the reference 531 base pair FV3 MCP target sequence. Nineteen of the 22 sequences had nucleic acid concentrations near or within the target DNA concentration range for sequencing (20-50 ng/μL) (A. Parker, pers. comm.).

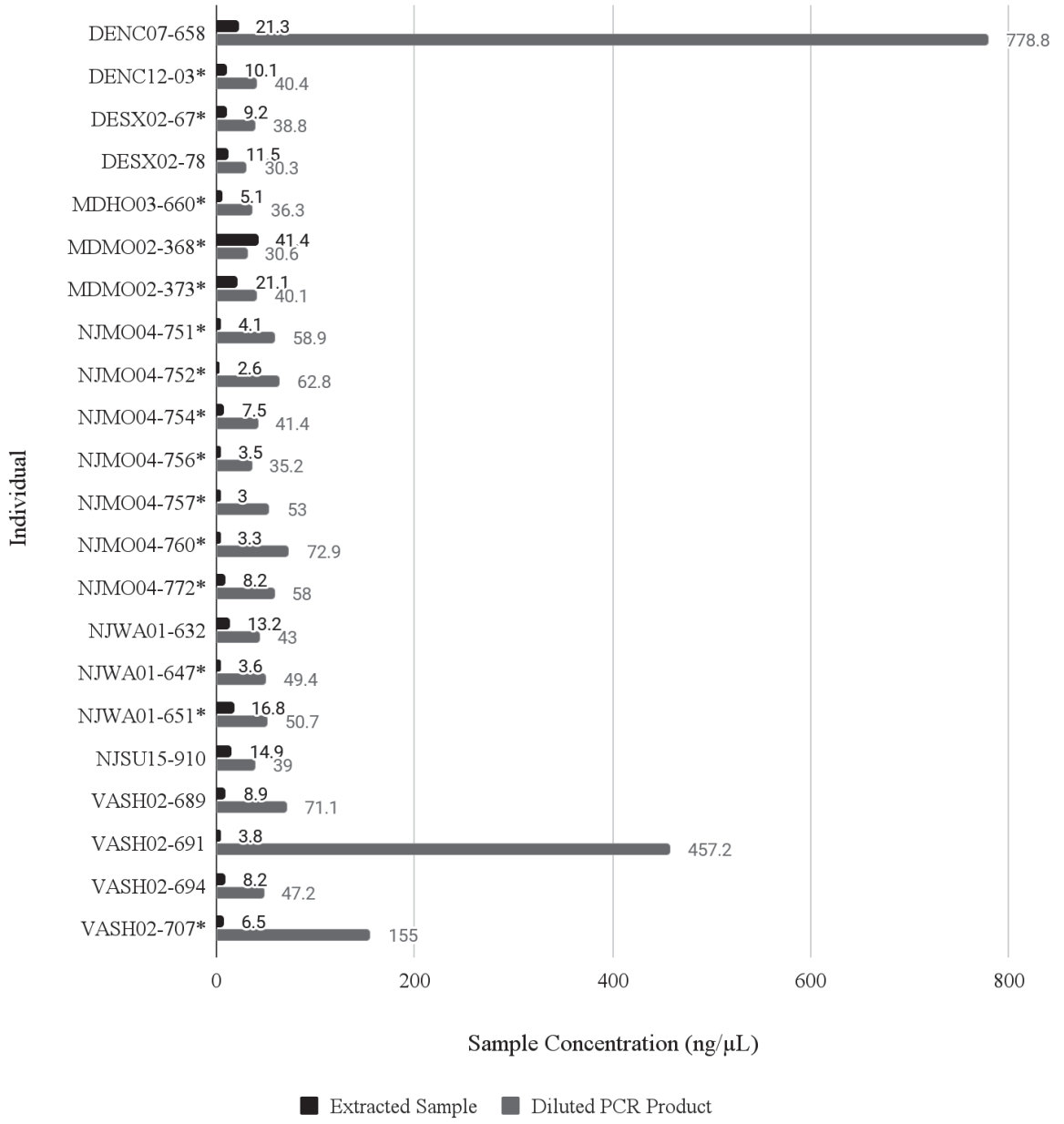

Figure 4: Extracted genomic DNA sample concentration (ng/µL) vs. diluted PCR product concentration (ng/µL)
The graph shows the measured amount of nucleic acids present in ng/µL for each sample that was sequenced. Measurements were taken for non amplified samples as well as after it was performed and diluted one ten-fold of the original PCR product.
* FV3 Sequence obtained

Measuring Protein and Other Contaminants in Sequencing Samples

In order to measure contamination factors that may affect the quality of sequencing results the 260/280 and 260/230 purity ratios of each sample were recorded using the NanoDrop. The 260/280 ratio is used to measure the purity of nucleic acid samples. As described by the Nanodrop manual (https://tools.thermofisher.com/content/sfs/manuals/nd-1000-v3.8-users-manual-8%205x11.pdf), a ratio of ~1.8 is typically accepted as a "pure" DNA sample while a ratio of ~2.0 is accepted as a "pure" RNA sample. Ratios below 1.8 for a DNA sample may indicate the presence of unwanted proteins or other contaminants which typically absorb strongly at approximately 280 nm. The 260/230 ratio is a secondary measure of nucleic acid purity. The most common range for pure samples is 1.8-2.2. Ratios below 1.8 may indicate the presence of co-purified contaminants that absorb at 230 nm such as regents used for Genomic DNA extraction including EDTA and guanidine hydrochloride. Other contaminants may possibly interfere with PCR and sequencing include pigments and environmental contaminants present on the tissue samples such as pollen, leaf litter/tannins, fungal spores, and bacteria along with contaminants that may be found within the kidney and liver tissue (Schrader et al., 2012 and references within). Figure 5 shows the ratios for all 22 samples that were sequenced. All samples noted with * had complete or nearly complete sequencing results that matched the reference 531 base pair FV3 MCP target sequence. The tenfold diluted PCR product 260/280 ratios ranged from 1.75 to 1.99. The tenfold diluted PCR product 260/230 ratios ranged from 1.9 to 2.23. The tenfold diluted PCR products provided similar or improved purity values when compared to the undiluted PCR product (Supplementary Table 1). The tenfold diluted PCR products provided the highest quality sequence results

compared to undiluted PCR product sequence quality values (Table 1 and Supplementary Table 2).

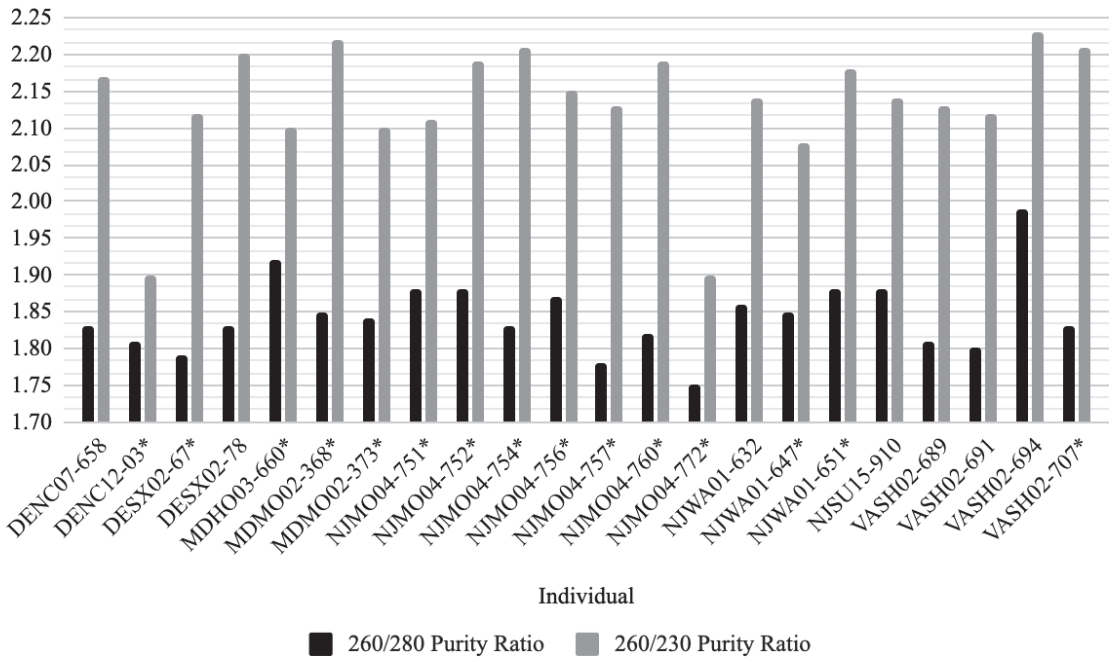

Figure 5: Tenfold diluted PCR product 260/280 and 260/230 purity ratios for all individuals that were cycle sequenced and analyzed.
* FV3 MCP target sequence obtained

Evaluation of Base Mutation

One mispair was located in two individuals from population NJMO04 (Figure 2 a&b). Upon further inspection the mispair appeared to be due to the presence of dual peaks for cytosine and thymine. Regions for individuals from the New Jersey population originally omitted from alignment due to poor quality were also reviewed (Figure 6). It appears a peak for thymine is present in reads for 6 individuals from the New Jersey population at the 372 base position. Peaks that were not recognized by the SnapGene software were noted with “N” indicating one or two nucleotides appears to be present at the 372 position. The peaks for thymine at the 372 position

do appear to be legitimate. Read one for individual 751 (Figure 6a) had two legitimate peaks likely causing the low quality value of 9 while read two had an overall poor quality reading and the peaks were not recognized by the Snapgene software at this location. The thymine peak at this location in read one appeared to be higher than that of the cytosine. Reads one and two for individual 752 (Figure 6b) both had legitimate peaks for cytosine and thymine at this location with quality values of 25 and 26, respectively. The cytosine peak is higher than that of the thymine for both reads. Read one for individual 754 (Figure 6c) has nearly identical peaks for both bases with a quality value of 12 while the overall quality of read two at this location compromised the quality of the peaks. Read one for individual 756 (Figure 6d) had a high quality peak for cytosine with a minor peak for thymine at the same location. The location of interest in read two for individual 756 was within a region of overall poor quality. The location in question appeared to show a prominent peak for cytosine with no thymine peak with a peak quality value of 7. Individual 757 (Figure 6e) had one reliable read with a base call at the area of interest for cytosine. The thymine peak was much less defined and appears to be associated with background noise. Read one and two for individual 760 (Figure 6f) show prominent peaks for cytosine with qualities of 38 and 62, respectively. Read one has a slight peak for thymine that is not well defined while read two does not show evidence of a thymine peak. Read one for individual 772 (Figure 6g) shows a prominent peak for cytosine with a lower but defined peak for thymine. Read two shows a prominent peak for cytosine with a minor less defined peak for thymine. The quality values for reads one and two were 32 and 43, respectively. The results of this analysis are summarized in Table 2. The GC content for the sequence containing cytosine is 58.6% while the sequence containing thymine is 58.4%.

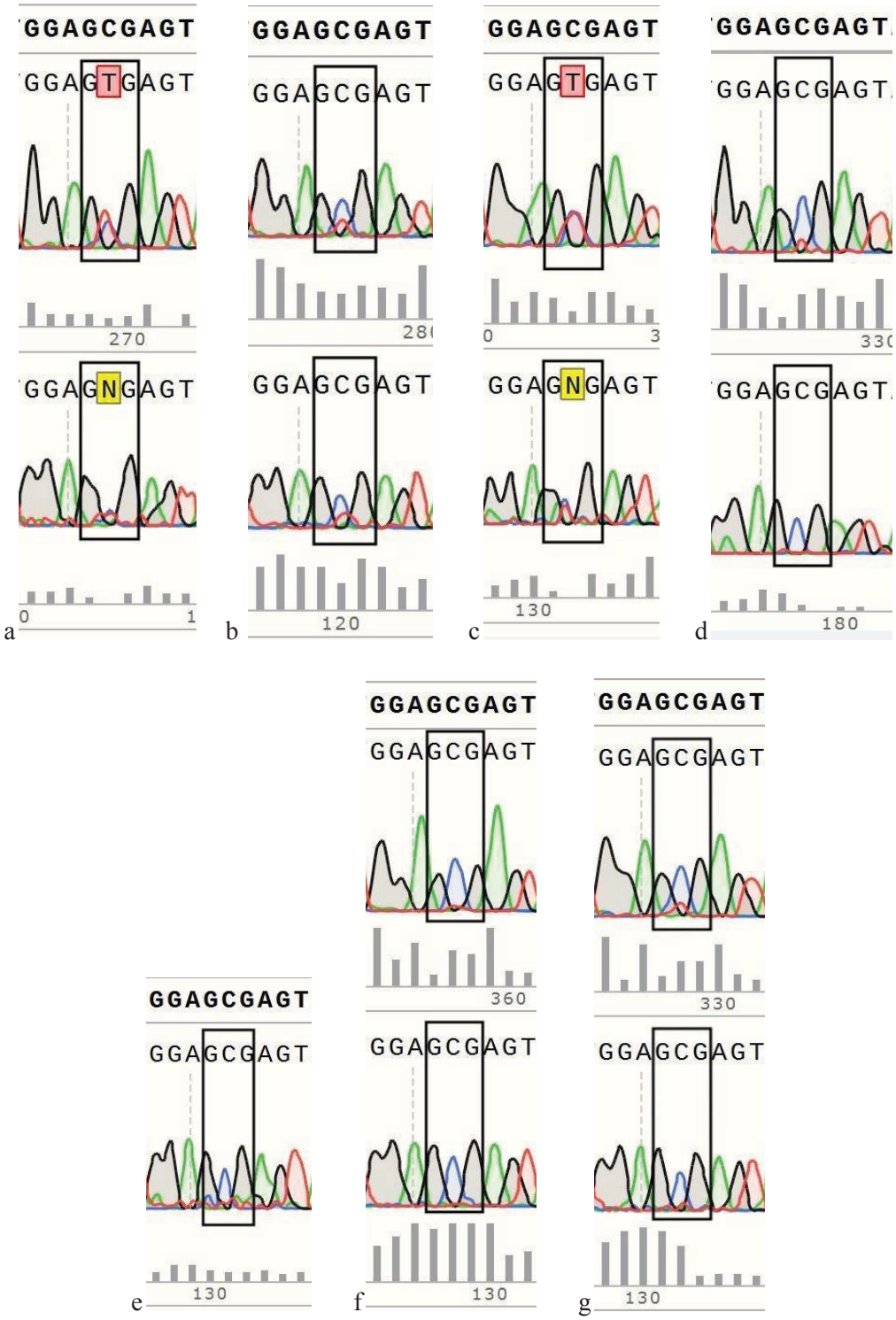

Figure 6: Evidence of variants present in population NJMO04
(a) Individual 751: top R1 quality value (QV) of peak: 9, and bottom R2 QV: peaks were not recognized by the SnapGene software, uncalled base was noted with N (nucleotide); (b) individual 752 top R1 QV: 25, bottom R2 QV: 26; (c) individual 754 top R1 QV:12, bottom R2 QV: peaks were not recognized by the SnapGene software, uncalled base was noted with N (nucleotide); (d) 756 top R1 QV: 37, bottom R2 QV: 7 (e) individual 757 top R1 QV: 10 (f) individual 760 top R1 QV: 38, bottom R2 QV: 62 (g) individual 772 top R1 QV: 32, bottom R2 QV: 43

Individual from NJMO04	DNA Concentration (ug/µL)	260/280 Purity Ratio	260/230 Purity Ratio	Read	Presence of Cytosine (C) / Thymine (T)	Quality Value of Peak for Nucleotide 372	Peak Height for Nucleotide 372
751	58.9	1.88	2.11	Read 1	C & T Present	9	106
				Read 2	C & T Present	*	*
752	62.8	1.88	2.19	Read 1	C & T Present	25	428
				Read 2	C & T Present	26	496
754	41.4	1.83	2.21	Read 1	C & T Present	12	306
				Read 2	C & T Present	*	*
756	35.2	1.87	2.15	Read 1	C & T Present	37	346
				Read 2	C Present	7	40
757	53	1.78	2.13	Read 1	C Present, T Inconclusive	10	416
760	72.9	1.82	2.19	Read 1	C & T Present	38	217
				Read 2	C Present, T Absent	62	645
772	58	1.75	1.9	Read 1	C & T Present	32	541
				Read 2	C Present, T Inconclusive	43	428

Table 2: Nucleotide Presence at Base Position 372
*Peaks not recognized by the SnapGene Software

Evaluation of Variant Amino Acid Sequence

In order to evaluate the functional effect of the thymine mutation on the MCP the amino acid sequence of the targeted region of the MCP gene was compared to the amino acid sequence with the base pair change of cytosine to thymine (Figure 7). The amino acid sequence for the targeted region of the FV3 MCP gene was obtained from the GenBank database (Mao et al.,

1996b, AC U36913) to ensure the read began at the correct frame. This analysis indicated that the change in base pair did not change the amino acid at that location. The amino acid sequence is not changed when this mutation is present. This is based on the assumption that there are no other mutations present in the poor quality regions of the query sequences.

```
FV3Reference        DLATYDNLERAMYGGSDATTYFVKEHYPVGWFTKLPSLAAKMSGNPAFGQQFSVGVPRSG
Variant             DLATYDNLERAMYGGSDATTYFVKEHYPVGWFTKLPSLAAKMSGNPAFGQQFSVGVPRSG
                    ************************************************************

FV3Reference        DYILNAWLVLKTPEVELLAANQLGDNGTIRWTKNPMHNIVESVTLSFNDISAQSFNTAYL
Variant             DYILNAWLVLKTPEVELLAANQLGDNGTIRWTKNPMHNIVESVTLSFNDISAQSFNTAYL
                    ************************************************************

FV3Reference        DAWSEYTMPEAKRTGYYNMIGNTSDLINPAPATGQDGARVLPAKNLVLPLPFFFSRD
Variant             DAWSEYTMPEAKRTGYYNMIGNTSDLINPAPATGQDGARVLPAKNLVLPLPFFFSRD
                    ***********************************************************
```

Figure 7: CLUSTAL multiple amino acid sequence alignment by MUSCLE (3.8)
Comparison of the sequence containing the thymine mutation vs. the reference sequence indicates that there is no alteration to the amino acid sequence when the mutation is present.

Discussion

Amplification of Target DNA using Primers MCP4 and MCP5

The results of the gel electrophoresis analysis indicated that 41 individuals from nine populations had amplification of a DNA fragment that was approximately 500 base pairs long (Figure 1). The length of these fragments matched the positive control length providing further support that the amplified DNA was from the primer targeted sequence of the FV3 MCP gene. Fluorescent levels varied among the samples; a number of factors could be attributed to the varying fluorescence. Strong fluorescent bands may be due to a large presence of PCR product, the addition of excess PCR product due to loading error, and the quality of the agarose gel. Initial viral load in the tissue for each individual is also a likely contributing factor to the varying levels of fluorescence. Additionally, no standard tissue size was established for genomic DNA extraction likely contributing to the variations in viral DNA concentrations in the genomic DNA samples. Measurement of the nucleic acid concentrations in genomic DNA samples would not provide adequate viral load data as additional genomic DNA from the host and any other DNA contaminants such as bacterial DNA found in the tissue samples would contribute to the overall DNA concentrations for each sample. Additionally it is important to note that traditional PCR and gel electrophoresis are not as sensitive as RT-PCR. It is possible the fluorescence observed during RT-PCR was not reflected in these findings due to lower viral loads and the use of methods with low sensitivity. A combination of the factors mentioned may also contribute to these results. Four positive controls derived from different FV3 genomic DNA extractions were used throughout the course of these experiments. The variation in positive control sample may account for the variations in fluorescent intensity for the controls.

Genetic Analysis

The results of the BLAST alignment show that the samples have an 84.73 to 99.79% match to the FV3 MCP gene targeted region (Supplementary Table 3). All 15 samples had at least one read that had percent identity values >91% with the aligned reference sequence indicating those individuals were in fact positive for FV3 *Ranavirus* (Figure 2, Supplementary Table 3) .

Confirmation of FV3 presence in two individuals from population MDMO02 further supports previous findings from the Monsen-Collar lab, presented in Smith et al. 2016, in which one individual successfully amplified with Traditional PCR. According to the previous study the individual was a 99% match to FV3 *Ranavirus* (Smith et al. 2016). According to the report, the gene sequence had a single nucleic acid base pair difference from the FV3 positive control sequence at nucleotide 540. The study concluded that the Maryland sample had an adenine base in place of a guanine base. My analysis of two additional individuals from the same population did not result in the same nucleic acid base pair difference. The sequences I obtained did not contain bases for the region in which the mutation was previously found, therefore the status of this mutation in individuals 368 and 373 from the Maryland population is unknown (Figure 2).

Measuring Nucleic Acid Concentration

Concentration measurements were collected after sequencing due to time constraints and to conserve the PCR product sample volumes (Figure 4). The concentrations were calculated using Beer's Law and are based on the concentration of absorbance at 260 nm. Results for individuals 658 and 691 from populations DENC07 and VASH02 were the product of evaporation due to poor sealing with plate tape causing excessively high concentration values of 778.8 and 457.2. High nucleic acid concentration levels (>50 ng/µL) were likely the dominant

factor that contributed to the poor sequence quality and lack of reference sequence coverage observed in Table 1 and Figure 3, respectively. In addition, nanodrop results do not indicate level of DNA and RNA, only total nucleic acids present. It is possible that these measurements included concentrations of RNA contaminants. Future analysis should utilize a Fluorometric DNA quantitation instrument to obtain a more accurate measure of DNA concentrations of genomic DNA prior PCR. PCR product concentrations should also be measured prior to sequencing in an effort to provide samples with DNA concentrations within the optimal range of 20-50 ng/µL (A. Parker, pers. comm.)

Measuring Protein, phenol and Other Contaminants in Sequencing Samples

The average quality values for the sequenced samples were not ideal. Values within the range of 40-60 represent the lowest possibility of incorrect base calls. In an effort to understand the factors that may affect sequence quality to improve future experimentation, the presence of non nucleic acid contaminants in the samples used for sequencing were evaluated (Figure 5). The 260/280 ratio is used to measure the purity of nucleic acid samples. As described by the Nanodrop manual, a ratio of ~1.8 is typically accepted as a "pure" DNA sample while a ratio of ~2.0 is accepted as a "pure" RNA sample. Ratios below 1.8 for a DNA sample may indicate the presence of unwanted proteins or other contaminants which typically absorb strongly at approximately 280 nm. Three samples sent for sequencing had values below 1.8, ranging from 1.75-1.79 (Figure 5 & Supplementary Table 1). Based on this minor deviation from the ideal value of 1.8 the level of protein contamination is likely low for those three samples and likely did not contribute to significant quality reduction. Ratios above 1.8 may indicate the detectable levels of RNA in the sample or contaminating proteins. Based on the results of this analysis 18 samples were >1.8 and as high as 1.99 (Figure 5 & Supplementary Table 1). This indicates there

may be low levels of RNA in the samples. The presence of RNA may interfere with the sequencing process by negatively affecting polymerase binding and amplification or extension (ThermoFisher Sequencing Handbook, https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/sequencing_handbook_FLR.pdf). The possible presence of RNA may contribute to poor sequence quality and overall low reference sequence coverage observed in Table 1 and Figure 3, respectively.

The 260/230 ratio is a secondary measure of nucleic acid purity. The most common range for pure samples is 1.8-2.2. Ratios below 1.8 may indicate the presence of co-purified contaminants that absorb at 230 nm such as regents used for Genomic DNA extraction including EDTA and guanidine hydrochloride (ThermoScientific, T042 Technical Bulletin, https://dna.uga.edu/wp-content/uploads/sites/51/2019/02/Note-on-the-260_280-and-260_230-Ratios.pdf). Other contaminants that may affect the overall purity of the DNA samples include pigments and environmental contaminants present on the tissue samples such as pollen, leaf litter/tannins, fungal spores, and bacteria along with contaminants that may be found within the kidney and liver tissue (Schrader et al., 2012 and references within). Results of this analysis show the samples used for sequencing had 260/230 ratios ranging from 1.9-2.23 indicating there was not a substantial amount of co-purified contaminants present that would potentially compromise the quality of the sequencing runs (Figure 5 & Supplementary Table 1).

Evaluation of Base Mutation

A single nucleic acid base pair difference was identified in six individuals from population NJMO04 (Figure 6). At the 372 base location of the reference sequence two peaks are visible, one for cytosine and the other for thymine. The peak height for both bases varies per sample. I also suspect that the overall lower quality of the peaks at this location is due to the

presence of the two bases which one would expect to reduce the sequence software's confidence with its analysis of the sequencing run. Sequence results for individuals 751, 752, 754, 756, 760, and 772 exhibit defined peaks for thymine that do not appear to be background noise. Peak heights and quality vary for each individual. The lack of uniformity of the thymine peak indicates its presence varies per individual, reducing my suspicion that it is due to background noise or contamination.

PCR product DNA concentrations for the individuals exhibiting this mutation ranged from 35.2-72.9 ng/µL (Figure 4). Those with concentrations within the recommended range of 20-50 ng/µL included individuals 754 and 756. These two individuals did not exhibit peak heights and quality values at the 372 positions that were drastically different from those of the other samples that had concentrations >50 ng/µL. The 260/280 values for the six NJMO04 samples ranged from 1.75-1.88 (Figure 5). Values lower than 1.8 may indicate a low level of protein contamination while values greater than 1.8 may indicate RNA contamination. Although the deviance from the 1.8 DNA purity value is minor (-.05 to +.08) protein and RNA contamination should not be ignored. Improved DNA purification methods should be employed for further analysis of these samples. The 260/230 values for the six samples ranged from 1.9-2.21 indicating that co-purified contaminants were not a substantial contributor to the overall read quality for these samples. Based on this analysis the mutation appears to be legitimate. The presence of excessive contaminants or background noise that often contribute to misreads do not appear to be a factor that would cause this phenomenon.

Due to the presence of these two prominent peaks it appears that sequence results for those samples harbored evidence of the presence of two different amplified MCP gene target regions. One sequence contained a cytosine base at the 372 base pair of the targeted region while

the second contained a thymine base. The presence of these peaks indicates the presence of an emerging variant in the population with a cytosine to thymine mutation while a version of FV3 without that mutation is also present. Peak heights for each nucleotide vary likely due to differences in the amount of viral load for each variant. In samples with higher cytosine peaks it is likely that the viral load for that FV3 variant was larger than the variant with the thymine mutation. Samples with equal peak height likely had equal viral loads for each variant. Based on this analysis of the individuals from the New Jersey population I suspect this was an emerging variant in this population. Further analysis of additional DNA samples from individuals that are positive for FV3 *Ranavirus* from this population is needed to further assess the prominence of this variant.

The GC (guanine/cytosine) content for the sequence containing cytosine is 58.6% while the sequence containing thymine is 58.4%. GC content dictates the melting temperature of DNA due to the presence of an additional hydrogen bond when compared to the bond between adenine and thymine. This difference in GC content is minor and likely does not contribute greatly to the melting temperature (Tm) discrepancy observed for many of the samples when compared to the positive controls prior to this study. The variability in Tm may be attributed to additional mutations that were not apparent in this data due to poor quality and incomplete sequence coverage. Improved sequence results may provide further insight into the causes of this phenomenon.

Evaluation of Variant Amino Acid Sequence

In order to assess the nature of the cytosine to thymine mutation the FV3 MCP nucleic acid sequence containing thymine at position 372 was transformed into an amino acid sequence (Figure 7). The variant amino acid sequence was aligned with the control amino acid sequence.

The thymine mutation did not affect the amino acid sequence indicating that this is a silent mutation. The presence of a silent mutation indicates that there was no alteration to the function of the MCP, thus, the fitness of this FV3 variant is not affected by this mutation.

Conclusion

Ultimately the findings of this analysis indicate 41 individuals from nine distinct populations previously deemed false positives exhibit amplified DNA evidence of a ~500 base pair sequence that is consistent with gel electrophoresis results for FV3 positive samples. Of those 41 individuals 22 were sequenced and 15 were confirmed positive for FV3. A cytosine to thymine mutation was located in individuals from population NJMO04 which did not affect the function of the FV3 MCP. These results indicate that the FV3 diagnostic standards for the Monsen-Collar Lab are not inclusive enough. The +/- 2 °C melting temperature threshold compared to the positive control may be too conservative as it does not account for emerging variants. Due to the difference in the structures of cytosine and thymine, mutations of this nature affect the melting temperature of the DNA. Mutations within the targeted region of the FV3 MCP gene may alter the melting temperature which can contribute to additional false positive results. With the current parameters in place, melting temperature changes >+/- 2 ℃ result in a negative diagnosis despite any evidence of DNA amplification during RT-PCR analysis. I advise the Monsen-Collar lab to further evaluate the prominence of mutations in Northeastern US wood frog FV3 infections. Increasing the melting temperature threshold may be appropriate but further analysis is needed to evaluate the range increase needed for the current FV3 conditions in this region. Alterations of the Tm threshold may also impact the results of the false positive results that did not amplify to a detectable level through traditional PCR and gel electrophoresis. All samples deemed false positives should be reevaluated if the Tm threshold is altered. In addition to improving diagnostic accuracy, genetic relatedness among different *Ranavirus* strains plays an important role in understanding future disease outbreaks. The study of evolutionary relatedness and age of viral strains provides better insight into the virulence of the FV3 virus and possible resistance within the host.

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Supplementary Materials

>FV3MCP
GACTTGGCCACTTATGACAATCTTGAGAGAGCAATGTACGGGGGTTCGGACGCCACC
ACGTACTTTGTCAAGGAGCACTACCCCGTGGGGTGGTTCACCAAGCTGCCGTCTCTG
GCTGCCAAGATGTCGGGTAACCCGGCTTTCGGGCAGCAGTTTTCGGTCGGCGTTCCC
AGGTCGGGGGGATTACATCCTCAACGCCTGGTTGGTGCTCAAGACCCCCGAGGTCGAG
CTCCTGGCTGCAAACCAGCTGGGAGACAATGGCACCATCAGGTGGACAAAGAACCC
CATGCACAACATTGTGGAGAGCGTCACCCTCTCATTCAACGACATCAGCGCCCAGTC
CTTTAACACGGCATACCTGGACGCCTGGAGCGAGTACACCATGCCAGAGGCCAAGCG
CACAGGCTACTATAACATGATAGGCAACACCAGCGATCTCATCAACCCCGCCCCGGCC
ACAGGCCAGGACGGAGCCAGGGTCCTCCCGGCCAAGAACCTGGTTCTTCCCCTCCC
ATTCTTCTTCTCCAGAGAC

Supplementary Figure 1: FV3 MCP 531 base pair reference sequence
(Mao et al., 1996b, GenBank AC U36913)

	Extracted DNA			PCR Product			1:10 Dilution PCR Product		
Individual	DNA Concentration (ng/μL)	260/280 Purity Ratio	260/230 Purity Ratio	DNA Concentration (ng/μL)	260/280 Purity Ratio	260/230 Purity Ratio	DNA Concentration (ng/μL)	260/280 Purity Ratio	260/230 Purity Ratio
DENC07-658	21.3	1.8	0.75	**	**	**	778.8	1.83	2.17
DENC12-03*	10.1	1.4	0.4	378.1	1.81	1.98	40.4	1.81	1.9
DESX02-67*	9.2	1.25	0.3	406.3	1.81	2.17	38.8	1.79	2.12
DESX02-78	11.5	1.36	0.37	387.3	1.83	2.22	30.3	1.83	2.2
MDHO03-660*	5.1	1.51	0.22	332.1	1.84	2.17	36.3	1.92	2.1
MDMO02-368*	41.4	1.88	0.92	396.7	1.87	2.19	30.6	1.85	2.22
MDMO02-373*	21.1	1.8	0.73	403.4	1.83	2.12	40.1	1.84	2.1
NJMO04-751*	4.1	1.14	0.17	520.6	1.81	2.06	58.9	1.88	2.11
NJMO04-752*	2.6	1.78	0.12	503.4	1.82	2.16	62.8	1.88	2.19
NJMO04-754*	7.5	2.3	0.24	440.8	1.79	2.11	41.4	1.83	2.21
NJMO04-756*	3.5	2.15	0.18	501.9	1.81	2.05	35.2	1.87	2.15
NJMO04-757*	3	1.25	0.16	459.2	1.81	2.09	53	1.78	2.13
NJMO04-760*	3.3	1.46	0.14	774.6	1.79	2.18	72.9	1.82	2.19
NJMO04-772*	8.2	2.37	0.41	490.9	1.83	2.17	58	1.75	1.9
NJWA01-632	13.2	1.54	0.57	385.5	1.83	2.21	43	1.86	2.14
NJWA01-647*	3.6	2.53	0.18	424.6	1.83	2.17	49.4	1.85	2.08
NJWA01-651*	16.8	1.96	0.59	410.9	1.83	2.16	50.7	1.88	2.18
NJSU15-910	14.9	1.52	0.38	389.4	1.83	2.24	39	1.88	2.14
VASH02-689	8.9	2.63	0.34	698.2	1.79	2.21	71.1	1.81	2.13
VASH02-691	3.8	1.29	0.19	**	**	**	457.2	1.8	2.12
VASH02-694	8.2	1.76	0.36	381.4	1.87	2.09	47.2	1.99	2.23
VASH02-707*	6.5	2.28	0.26	1382.1	1.75	2.2	155	1.83	2.21

Supplementary Table 1: Nanodrop results

* FV3 Sequence obtained

** No data available

Individual	Average Quality of Chromatogram	Total Sequence Length (base pairs)	Highest Quality Region Length (base pairs)
MDHO03 660	R1: 16.7 | R2: 19.3	R1: 439 | R2: 431	R1: 355 | R2: 390
NJMO04 752	R1: 14.0 | R2: 9.1	R1: 410 | R2: 368	R1: 317 | R2: 5
NJMO04 760	R1: 12.5 | R2: 9.0	R1: 506 | R2: 406	R1: 329 | R2: 6
NJMO04 772	R1: 16.1 | R2: 14.6	R1: 445 | R2: 421 b	R1: 382 | R2: 291
NJWA01 647	R1: 11.8 | R2: 9.7	R1: 395 | R2: 364	R1: 173 | R2: 5
NJWA01 651	R1: 11.2 | R2: 15.5	R1: 189 | R2: 419	R1: 5 | R2: 392

Supplementary Table 2: Quality values and sequence length for undiluted PCR Products
R1=Forward Primer Sequence Read
R2=Reverse Primer Sequence Read

	R1 E-value	R1 Per. Identity	R2 E-value	R2 Per. Identity	R3 E-value	R3 Per. Identity	R4 E-value	R4 Per. Identity
DENC07 658	*	*	*	*				
DENC12 03	0	97.22%	**	**				
DESX02 67	0	97.52%	*	*				
DESX02 78	**	**	**	**				
MDHO03 660	0	99.58%	4.00E-124^	87.03%^				
MDMO02 368	0	99.79%	0	99.16%	0	98.98%	0	99.16%
MDMO02 373	0	98.95%	0	99.16%				
NJMO04 751	1.00E-82	84.73%	0	98.58%				
NJMO04 752	1.00E-123	93.09%	0	96.57%				
NJMO04 754	1.00E-174	91.76%	2.00E-146	87.37%				
NJMO04 756	0	96.52%	*	*				
NJMO04 757	**	**	0	96.26%				
NJMO04 760	0	96.94%	0	99.15%				
NJMO04 772	0	96.70%	0	97.88%				
NJWA01 632	*	*	**	**				
NJWA01 647	2.00E-176	95.14%	0	98.59%				
NJWA01 651	0	99.35%	*^	*^				
NJSU15 910	6.00E-71	91.98%	**	**				
VASH02 689	*	*	*	*				
VASH02 691	*	*	*	*				
VASH02 694	*	*	*	*				
VASH02 707	3.00E-170	90.13%	*	*				

Supplementary Table 3: BLAST alignment of query sequences with 531 base pair reference FV3 MCP target sequence

*No significant similarity found

**Not enough sequence to work with

^R2 of undiluted PCR product

Individual	Number of Base Pairs Aligned with Reference Sequence	Percent Alignment with Reference Sequence (%)
DENC07 658	0	0
DENC12 03	386	72.69
DESX02 67	387	72.88
DESX02 78	0	0
MDHO03 660	455	85.69
MDMO02 368	529	99.62
MDMO02 373	531	100
NJMO04 751	486	91.53
NJMO04 752	412	77.59
NJMO04 754	506	95.29
NJMO04 756	362	68.17
NJMO04 757	385	72.50
NJMO04 760	531	100
NJMO04 772	522	98.31
NJWA01 632	0	0
NJWA01 647	531	100
NJWA01 651	442	83.24
NJSU15 910	0	0
VASH02 689	0	0
VASH02 691	0	0
VASH02 694	0	0
VASH02 707	302	56.87

Supplementary Table 4: Number and percent alignment of nucleotides for multi sequence alignment