Species Identification of Two Suspected Hybrid Salmonids in the North Fork Touchet River in Southeastern Washington State

Report by

Jessica Beach
California State University, Northridge
Northridge, CA 91330-8207

and

Jennifer F. Von Bargen and Scott M. Blankenship
WDFW Molecular Genetics Laboratory
600 Capitol Way N, Olympia, WA 98501-1091, USA

Introduction

Hybridization is the process in which two genetically distinct species breed and produce an offspring that is commonly known as a hybrid. This phenomenon has been observed in nature on many instances and with a variety of plants and animals. Some common examples of hybrids include the mule (cross between a horse and a donkey) and peppermint (a hybrid between spearmint and water mint). What is the significance of hybridization? Some scientists contend that natural hybridization has lead to the production of relatively fit hybrids that possess novel genetic variation or found new evolutionary lineages (Arnold and Hodges 1995). Additionally, studies have demonstrated that some hybrid species are more fit than either parental species. This increased fitness, due to the hybrids mixed genome complexes, may allow hybrids to develop new niches and adapt more readily to change (Cruzan and Arnold 1993). Conversely, others argue that a majority of hybrids are less fit than their parental species because they are sterile and unable to propagate their genetic information into future generations. This sterility, therefore, makes hybridization of little evolutionary significance (Dobzhansky 1970). Yet, fertile hybrids have been observed in nature, thereby providing a mechanism for gene flow between species. Additionally, if the gene flow is sufficiently large, hybridization may lead to the merging of two formally distinct species.

Fish hybridization is observed more frequently in the wild than any other group of vertebrates (Ryman & Utter 1987). Examples include natural hybridization between Dolly Varden and bull trout (Baxter et al. 1997) and crosses between steelhead trout and costal cutthroat trout (Campton & Utter 1985). Several factors contribute to the increased rate of fish hybridization: (1) external fertilization; (2) weak ethological isolating mechanisms; (3) unequal abundance of two parent species; (4) competition for spawning habitat; and (5) susceptibility to secondary contact between recently evolved forms (Campton 1987).

Introduction of non-native fish species into freshwater habitats has also resulted in hybridization. In the western United States, the introduction of the rainbow trout into nonnative regions has resulted in the introgression of rainbow genes into the indigenous cutthroat population that is morphologically undetectable (Leary et al. 1984). This induced gene flow is a concern because it

may impact the integrity of native gene pools and ultimately result in the extinction of several freshwater species through introgression (Rubidge & Taylor 2005).

The present study was precipitated by an instance of possible hybridization, when a Washington Department of Fish and Wildlife (WDFW) biologist collected two salmonids while conducting fieldwork in southeastern Washington State (North Fork Touchet River) that could not be identified to species (Figure 1). When keyed, these fish had several characteristics of rainbow trout, and were identified as likely rainbow trout, except their coloration was unusual (i.e. dirty brown) and they lacked parr marks (Glen Mendel, pers. comm.). The coloration exhibited was darker then that observed for any indigenous salmonid species suggesting possible hybridization. Indigenous salmonids include steelhead/redband trout, bull trout, and Chinook salmon. Non-native rainbow trout have been introduced, as have brown trout. Coho salmon reintroductions have occurred elsewhere in the Columbia River Basin. It is possible that Atlantic salmon have escaped from aquaculture facilities and dispersed into the area where these unknowns were found. The primary objective of this project was to use genetic analyses to determine if the unknown salmonids were of hybrid origin. Mitochondrial DNA was used to establish the species of the maternal lineage. Microsatellite analysis was used as an additional method of species identification and as a way to detect possible hybridization (i.e. the possession of a mixture of alleles from both parental species).

Materials and Methods

Sampling

Two salmonid specimens of possible hybrid origin were collected from the North Fork Touchet River in southeastern Washington and were maintained in 100% ethanol. Tissue standards from the following species were used as controls during mitochondrial genome sequencing: Chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*O. kisutch*), sockeye salmon (*O. nerka*), pink salmon (*O. gorbuscha*), chum salmon (*O. keta*), steelhead/rainbow trout (*O. mykiss spp.*), brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*), and bull trout (*Salvelinus confluentus*). In addition, comparison samples of brown and rainbow trout from the collection area (Tucannon

Hatchery) were also included. In the subsequent microsatellite analysis only brown and rainbow trout samples were used.

Extraction of DNA

DNA was extracted by using the NucleoSpin® 96 Tissue kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions for tissue preparations.

PCR Amplification

Polymerase chain reaction (PCR) was used to perform fragment analysis on both the mitochondrial cytochrome b gene and nuclear microsatellite genetic markers. The cytochrome b region of the mitochondrial genome was assessed using fourteen primer sets that amplified sections of the gene in an allele specific manner (Table 1) (WDFW unpublished). Species designation is inferred by the presence or absence of specific amplified fragments. Cytochrome b PCR reaction volumes were 10 µL, and consisted of 1.0 µL 10X PCR buffer (Promega), 0.60 µL $MgCl_2$ (1.5 mM final) (Promega), 1.0 µL 10mM dNTP mix (Promega), 0.10 µL (0.05 mM final) *Taq* DNA polymerase (Promega). All loci were amplified as a single set with an annealing temperature of 55ºC and used a 0.2 Molar solution of each primer. Thermal cycling was carried out on a PTC-200 thermal cycler (MJ Research) as follows: 94ºC (2 minutes); 30 cycles of 94ºC for 15 seconds, 55ºC for 30 seconds, and 72ºC for 1 minute; a final 72ºC extension for 30 minutes.

Microsatellite PCR amplification was performed using 20 fluorescently end-labeled microsatellite loci, *One* 101, 102, 108, and 114 (Olsen et al. 2000), *One* 18 (Scribner et al 1996), *Omy* 1001 and 1011 (Spies et al. 2005), *Omm* 1070, 1128, and 1130 (Rexroad et al. 2001), *Ots* 100 (Nelson and Beacham, 1999), *Ots* 103 (Beachman et al. 1998), *Sco* 102, 103, 104, 105, 106, 107, 109, and 110 (WDFW unpublished). PCR reaction volumes were 10 µL, and consisted of 1.0 µL 10X PCR buffer (Promega), 0.60 µL $MgCl_2$ (1.5 mM final) (Promega), 1.0 µL 10mM dNTP mix (Promega), 0.10 µL (0.05 mM final) *Taq* DNA polymerase (Promega). Eight multiplexed sets were used to amplify the loci, resulting in various primer molarities, annealing temperatures, and number of cycles (Table2). Thermal cycling was carried out on a PTC-200 thermal cycler (MJ Research) as follows: 94ºC (3 minutes); indicated cycles (Table 2) of 94ºC for 15 seconds, annealing

temperature (Table 2) for 30 seconds, and 72ºC for 1 minute; and a final 72ºC extension for 30 minutes.

PCR Product Visualization

PCR products were visualized by denaturing polyacrylamide gel electrophoresis on an ABI 3730 automated capillary sequencer (Applied Biosystems). Fragment analysis was conducted using GeneMapper 3.7 (Applied Biosystems) for both the mtDNA and microsatellite loci.

Results & Discussion

We report that genetic variation present for the mitochondrial cytochrome b gene and nuclear microsatellite markers are consistent with the suspect salmonid individuals having male and female rainbow trout parents. Mitochondrial DNA fragment analysis demonstrated distinct alleles amplified for each comparison control species. Both unknown salmonid samples possessed the same allele sequence, Oke-Sco-Omy-Oki-end marker. Comparison with the control mitochondrial samples indicated that this sequence was characteristic of steelhead and rainbow trout. The mitochondria is materially inherited, therefore this test is used to identify species of the maternal parent. Nuclear DNA markers (i.e. microsatellites) were subsequent used to infer the male parent.

Of the twenty microsatellite loci used, eight loci successfully amplified in all samples; six loci amplified successfully in the rainbow trout and unknown samples, but failed in all brown trout samples; and six loci completely failed to amplify. The eight successful loci are as follows: *Omy*-1001, *One*-18, *One*-108, *One*-114, *Ots*-100, *Sco*-103, *Sco*-105, and *Sco*-107. Of these eight, five loci demonstrated divergent allele ranges between brown trout and rainbow trout sampled to assist in species identification. At all five loci where species assignment was possible, the unknown samples assigned to the rainbow trout population. *Omy*-1001 (Figure 2) showed the brown trout alleles between the ranges of 72-77 base pairs, while the rainbow trout alleles ranged from 179-220 base pairs. The alleles for both unknown samples, ranging from 192-198 base pairs, fell within the rainbow trout population. *One*-18 (Figure 3) also showed the unknown sample alleles to fall within the rainbow trout allele range of 165-180 base pairs, while the brown trout alleles

ranged from 211-281 base pairs. Yet, it was also observed that the brown trout frequently possessed three alleles at this locus making comparison with the rainbow trout difficult. *One*-108 (Figure 4) displayed allele separation between brown and rainbow trout. Rainbow trout alleles ranged from 178-241 base pairs, while the brown trout had larger alleles in the range of 336-361 base pairs. The unknown alleles were observed to occur within the rainbow trout range at 181, 189, and 213 base pairs. At locus *Ots*-100 (Figure 6), the brown trout had a fixed allele at 124 base pairs, while the rainbow trout ranged from 169-215 base pairs. The alleles of the unknown samples were observed at 185 base pairs, within the middle of the observed rainbow trout alleles. Locus *Sco*-105 (Figure 8) showed fixed alleles, different in both the brown trout and rainbow trout populations. The brown trout alleles occurred at 135 base pairs, while the rainbow trout possessed alleles at 124 and 202 base pairs. Consistent with the rainbow trout population, the unknown samples also possessed alleles at 124 and 202 base pairs.

Loci *One*-114 (Figure 5), *Sco*-103 (Figure 7), and *Sco*-107 (Figure 9) did not display a significant degree of allele separation between brown trout and rainbow trout to allow for species identification. In all cases, there was a major overlap between allele frequencies. For example at *One-114*, the brown trout alleles were observed 226, 230, 238, and 242 base pairs, which was located within the rainbow trout allele range of 209-248 base pairs. In addition, at all three loci, the unknown samples possessed alleles that did not fall within the observed allele-range of either the rainbow trout or brown trout samples. This observation is most likely the result of a small sample sizes compared for brown and rainbow trout. A larger sample size would encompass greater genetic diversity for the species and may demonstrate that the unknown sample alleles are not outside known ranges. A more remote possibility is that the unknown samples are a hybrid between rainbow and another species. Yet, this is highly unlikely given that we observed allelic diversity at many loci characteristic of rainbow trout.

Circumstantially, it is also interesting to note that in loci where there was complete amplification failure of the brown trout samples and amplification of the rainbow trout samples, the unknown samples amplified successfully. These loci included: *Omm* 1130 and 1128, *One* 101 and 102, *Ots*-103, and *Sco*-104. This observation also suggests the unknown samples are most likely of the rainbow trout species.

Acknowledgements

I would like to thank Ken Warheit and all the staff of the WDFW Genetic Stock Identification Lab for allowing me this summer internship opportunity.

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Table 1 Mitochondrial cytochrome b PCR primers used for species identification.

Primers				
SpID-L10333 (F) V1	Ocl-H10382 (R)	Oke-H10425(R)	Ssa-H10653 (R)	Sco-H10537 (R)
SpID-H10678 (R)	One-H10576 (R)	Oki-H10676 (R)	Sal-H10469 (R)	V1
Ots-H10446 (R)	Omy-H10637 (R)	Ogo-H10585 (R)	Sfo-H10532 (R)	

Table 2 Multiplexed microsatellite primer sets.

Multiplex	Loci	Molarity (M)	Annealing Temperature	Cycles
Sco-A	Sco-109	0.25		
	Sco-104	0.05	57ºC	32
	Sco-107	0.04		
Sco-B	Sco-106	0.06	57ºC	32
	Sco-103	0.05		
Sco-C	Sco-110	0.05		
	Sco-102	0.04	57ºC	32
	Omm-1130	0.07		
Sco-E	Omm-1128	0.04	47ºC	30
	Sco-105	0.035		
Omy-E2	Omm-1130	0.05		
	Omm-1070	0.025	62ºC	26
	Omy-1011	0.045		
Omy-F2,1	Omy-1001	0.06		
	Omm-1128	0.05	52ºC	30
	One-18	0.04		
Omy-B2	One-102	0.10		
	One-114	0.10	55ºC	29
	Ots-100	0.04		
Omy-C2	One-108	0.08		
	Ots-103	0.03	55ºC	29
	One-101	0.05		

Figure 1. Two salmonids collected by Department of Fish and Wildlife (WDFW) biologist while conducting fieldwork in southeastern Washington State (N.F. Touchet River) that could not be identified to species – keyed as likely rainbow trout.

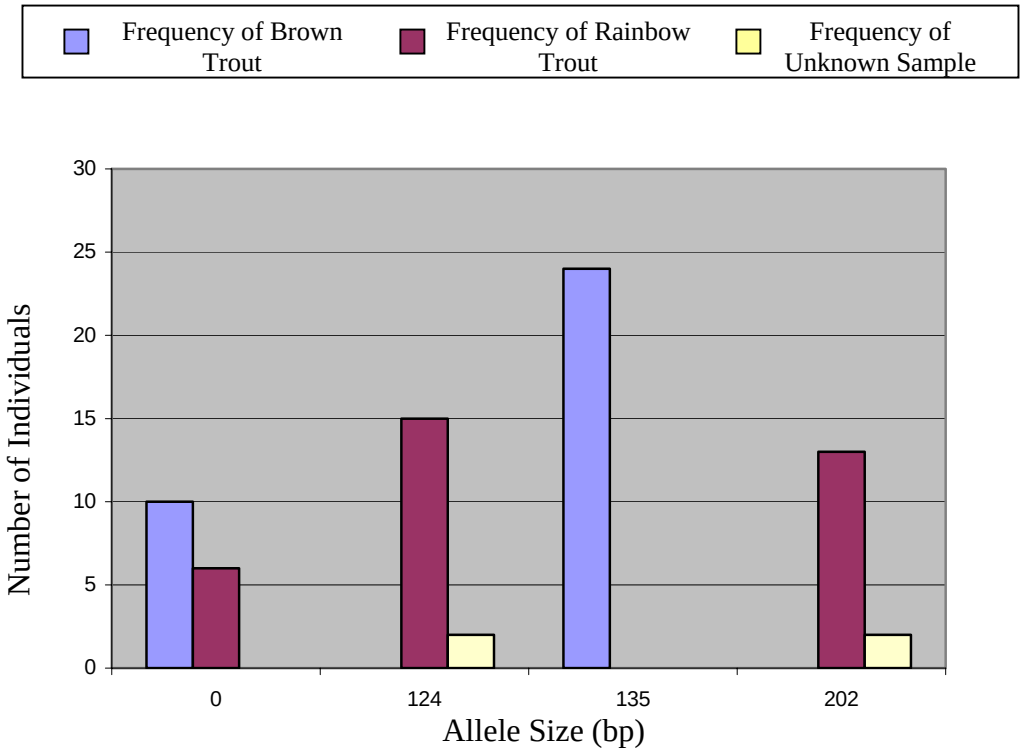

Figure 2. Allele frequency of *Omy*-1001

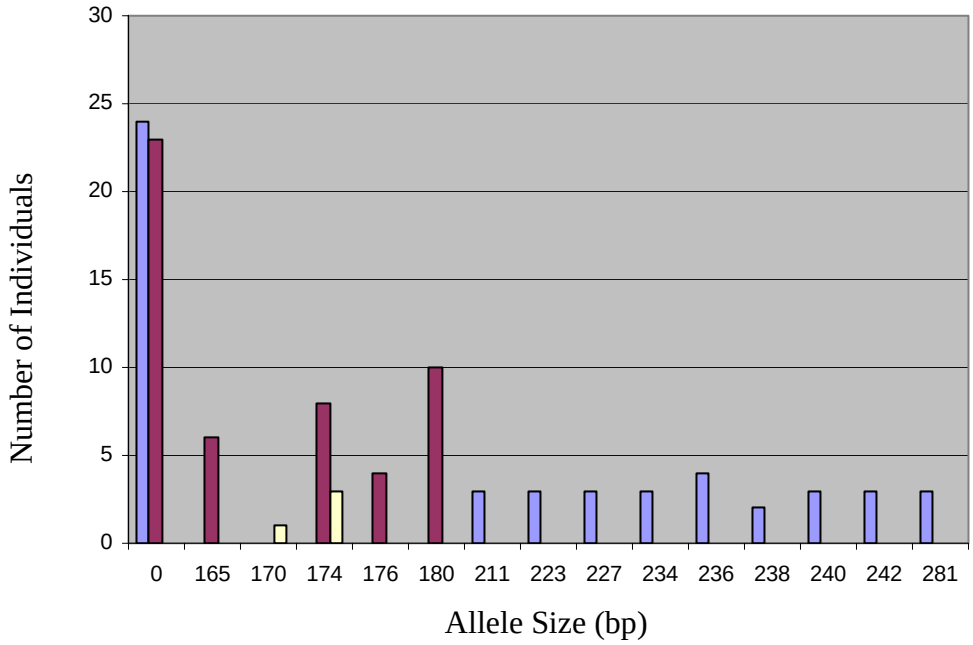

Figure 3. Allele frequency of *One*-18

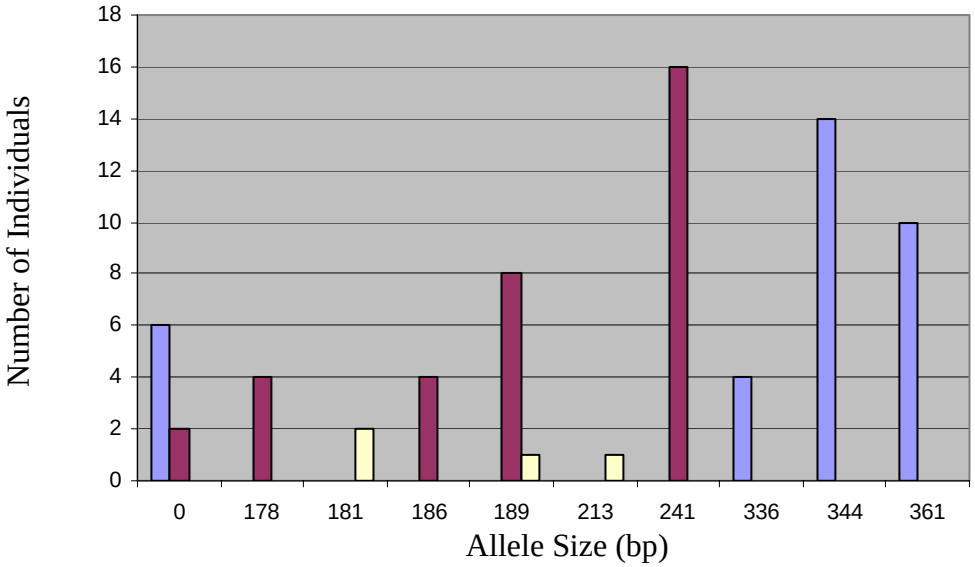

Figure 4. Allele frequency of *One*-108

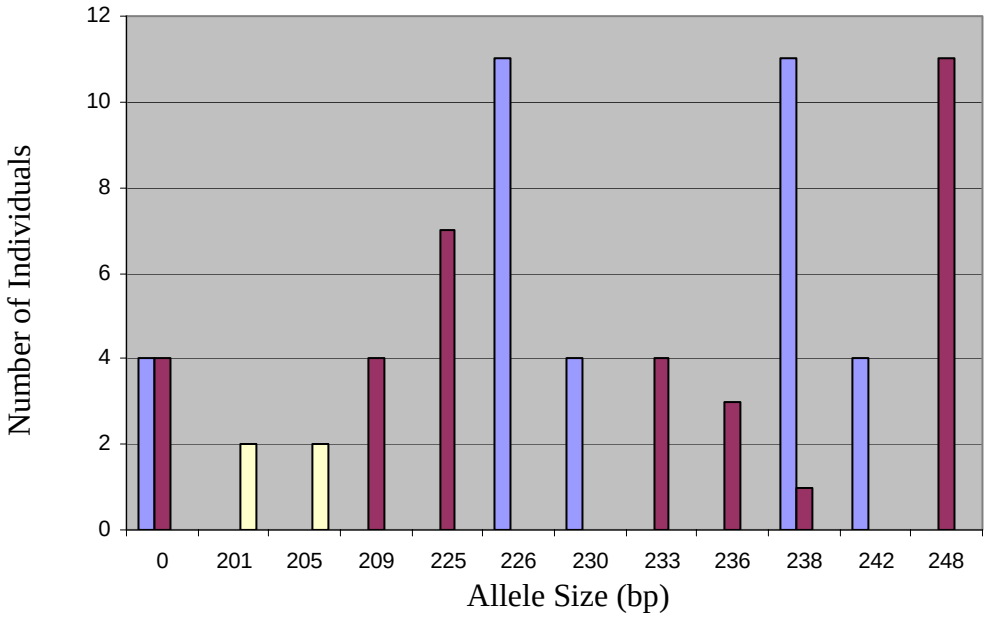

Figure 5. Allele frequency of *One*-114

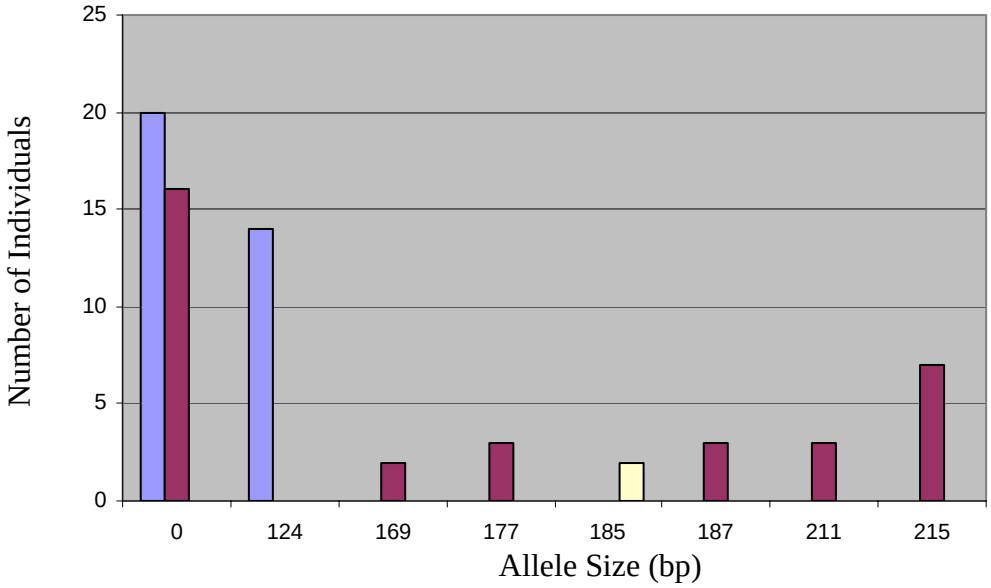

Figure 6. Allele frequency of *Ots*-100

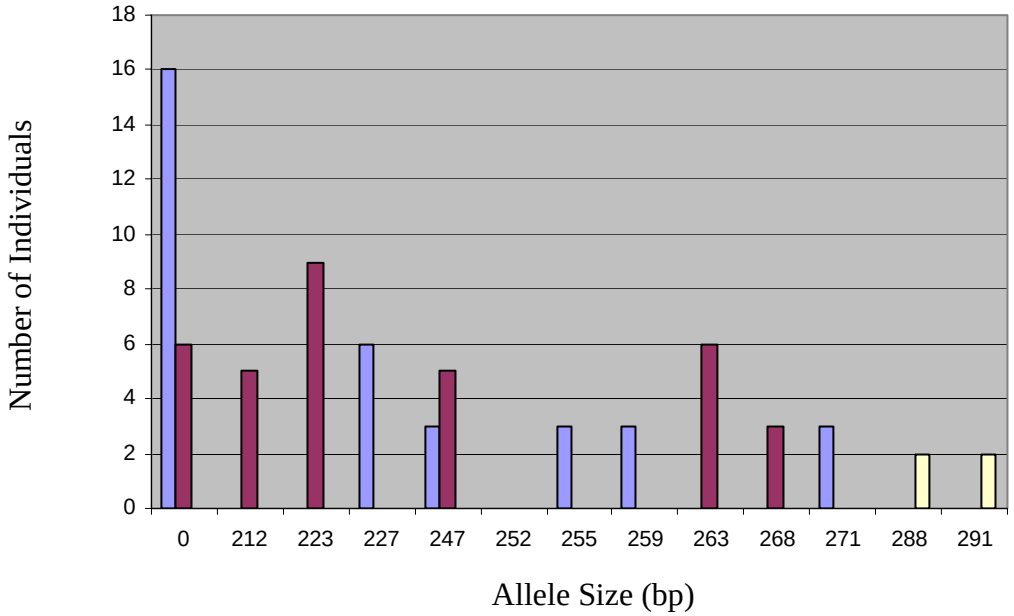

Figure 7. Allele frequency of *Sco*-103

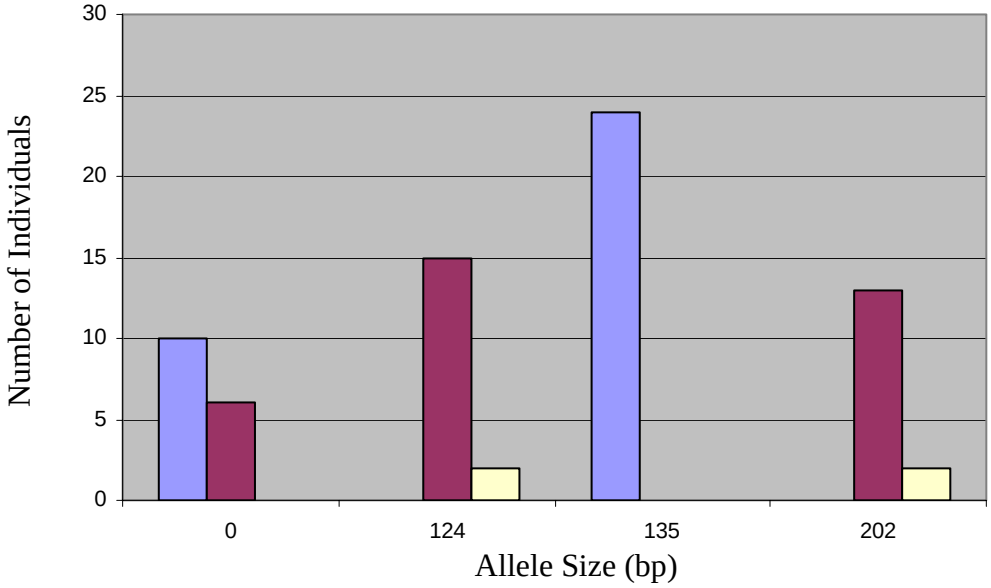

Figure 8. Allele frequency of *Sco*-105

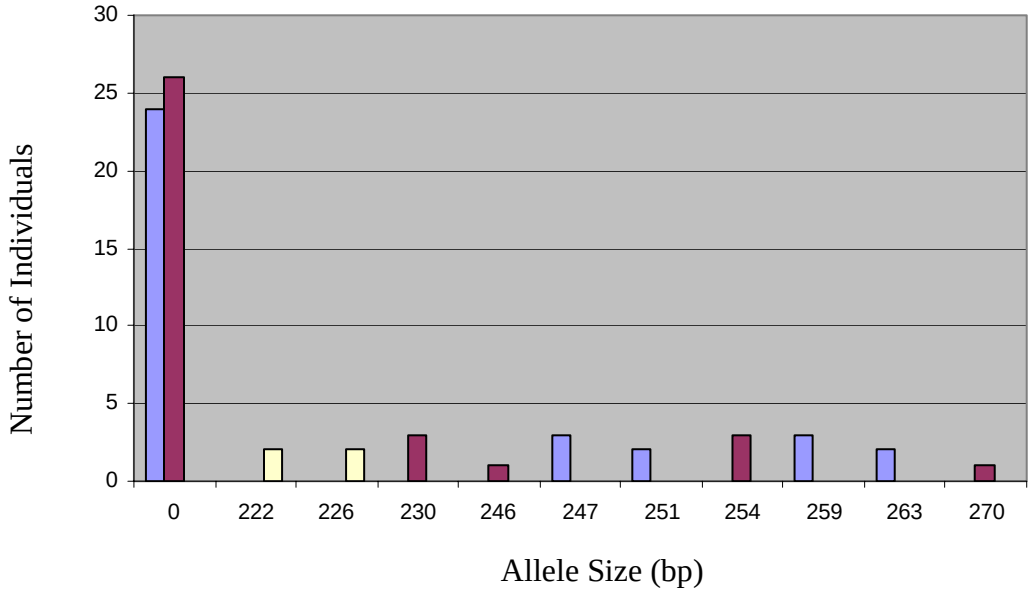

Figure 9. Allele frequency of *Sco*-107