DNA PROFILING OF CAPTIVE ROSEATE SPOONBILL (*Ajaia ajaja*) POPULATIONS AS A MECHANISM OF DETERMINING LINEAGE IN COLONIAL NESTING BIRDS

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Roseate Spoonbills are colonial nesting birds with breeding grounds extending from the United States Gulf coast to the pampas of Argentina. The U.S. population suffered a severe bottleneck from 1890 to 1920. The population's recovery was slow and partially credited to migrations from Mexican rookeries, but a gene pool reduction would be expected.

Five polymorphic Spoonbill autosomal short tandem repeat (STR) loci [three $(GAT)_n$, one $(AAAG)_n$ and one $(GT)_n$] and one Z/W-linked microsatellite exhibiting sex-specific dimorphism were isolated and characterized. The Z/W-linked STR locus accurately confirmed the sex of each bird. Allelic profiles for 51 Spoonbills obtained from Dallas (Texas), Fort Worth (Texas) and Sedgwick County (Kansas) zoos revealed a non-continuous distribution of allele frequencies, consistent with the effects of a population bottleneck. Allelic frequencies also differed significantly between the isolated zoo populations.

Although extra-pair copulations were suspected and difficult to document, zoos commonly used observational studies of mating pairs to determine familial relationships among adults and offspring. STR parentage analysis of recorded family relationships

excluded one or both parents in 10/25 cases studied and it was further possible to identify alternative likely parents in each case.

Mistaken familial relationships quickly lead to the loss of genetic variability in captive populations. Here, a decreased heterozygosity (H_O) in 2nd generation captive-bred birds was observed at 3 out of 4 loci evaluated. Although these results could not be statistically validated because of the small number of individuals available for study (15 wild birds with no offspring vs. eight 2nd generation captive birds), they are considered biologically important, as decreased H_O is an indicator of inbreeding and this apparent decrease occurred within two generations of removal from the wild. Collectively, the evidence obtained from this study suggests that captive Spoonbill populations are experiencing rapid loss of diversity from an already depleted wild gene pool.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

Ap	ampicillin
Ap^r	ampicillin resistance
bp	base pair
BSA	bovine serum albumin
°C	degrees centigrade
CIP	calf intestinal phosphatase
Ci	Curie
cm	centimeter
cpm	counts per minute
CsCl	cesium chloride
dnt	did not type
dNTP	deoxyribonucleotide
ddNTP	dideoxyribonucleotide
ddH_2O	distilled deionized water
DNA	deoxyribonucleic acid
dsDNA	double-strand DNA
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
g	gram
G	gauge
H_E	expected heterozygosity
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
H_O	observed heterozygosity
IPTG	isopropyl-β-D-thio-galactopyranoside
kbp	kilobase pair
Km	kanamycin
Km^r	kanamycin resistance
LB	Luria-Bertani
l	liter
LMP	low melting point
M	molar
mbp	megabase pair
mCi	milliCurie
MCS	multiple cloning site
MHC	Major Histocompatability Complex
min	minutes
ml	milliliter
mm	millimeter

mM	millimolar
mol	mole
mtDNA	mitochondrial DNA
µg	microgram
µl	microliter
NaCl	sodium chloride
ng	nanogram
NaOH	sodium hydroxide
nm	nanometer
N-PAGE	non-denaturing polyacrylamide gel electrophoresis
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque forming units
pg	picogram
pmol	picomole
RAPD	random amplified polymorphic DNA
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
sec	seconds
SNPs	single nucleotide polymorphisms
SSC	standard saline citrate
ssDNA	single-stranded DNA
STR	short tandem repeat
SSPE	sodium chloride, sodium phosphate, ethylenediamine tetraacetic acid
TAE	(hydroxymethyl) aminomethane, acetic acid, ethylenediamine tetraacetic acid
TBE	(hydroxymethyl) aminomethane, boric acid, ethylenediamine tetraacetic acid
Tc	tetracycline
Tc^r	tetracycline resistance
TE	(hydroxymethyl) aminomethane, ethylenediamine tetraacetic acid
TEMED	*N,N,N',N'*-tetramethylethylenediamine
Tris	(hydroxymethyl) aminomethane
U	units
UV	ultraviolet
V	Volts
VNTR	variable number tandem repeat
W	Watts
x g	times the force of gravity
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER I

INTRODUCTION

The management of wildlife populations has become increasingly important as their habitats are fragmented by human development and their ecological niches are invaded by exotic species. Traditionally, zoos have maintained various species in captivity in order to showcase their exotic nature and have acquired many of their captives by collection from wild populations. Current zoological practices are designed to limit the number of animals collected from the wild and efforts are made to support wild populations by re-introduction of endangered or threatened species where habitats are still suitable. To this end, many zoos are now trying to ascertain the usefulness of their captive populations to the extant wild populations and to maintain their captive populations without further decimation of already struggling wild populations via specimen collection.

The Fort Worth Zoo, in Fort Worth, Texas, has committed considerable time and resources in order to understand the genetics of the Roseate Spoonbill, *Ajaia ajaja*, a Gulf coast wading bird of which they have a captive population. Many of the Spoonbills in zoos throughout the United States originated from the Fort Worth Zoo (Chris Brown, personal communication), and it is thus appropriate to begin a genetic study with their present population of 22 individuals. This collection includes individuals representing many generations of captive breeding as well as specimens which were acquired from the wild which may thus harbor unique genetic characteristics. Blood samples from 22 Fort

Worth Zoo Spoonbills, 20 Dallas Zoo (Dallas, TX) Spoonbills and 9 Sedgwick County Zoo (Wichita, Kansas) Spoonbills were obtained for the purpose of isolating the DNA to be 1) tested for genetic markers, 2) subjected to parentage analyses, and 3) characterized to assess the genetic health of captive and wild Roseate Spoonbill populations. Tables IV, V, and VI (see Results and Discussion) list the Spoonbill DNA samples isolated and analyzed as part of this study.

Roseate Spoonbills are colonial nesting birds with breeding grounds extending from the Gulf coast of the United States to the pampas of Argentina. Prior to the 1850s, the U.S. Gulf coast population estimates of these birds were in the thousands. Throughout the late 1800s and early 1900s their breeding sites were severely disturbed by coastal development and by hunters killing egrets for the millinery trade (Allen, 1942). The period of 1890 to 1919 was the "low water mark" for the species in the United States. During this time there were approximately 3 small colonies totaling only 20 to 25 nests. The recovery of populations in Texas and Louisiana is credited to protective laws, warden-patrolled sanctuaries, and the decreased demand for feathers. By 1941 the summer population in the United States had exceeded 5,500 individuals (3,500 nonbreeding) of which 89% were in Texas. Even with these numbers, the breeding potential of the species was considered relatively low.

From a population genetics perspective the gene pool of the birds had been severely reduced during the period of 1890 to 1919, resulting in a phenomenon known as the population bottleneck effect. The severe reduction of the gene pool means there is less total genetic variation among the surviving individuals of the population, leaving fewer genetically distinct individuals and possibly the entire population vulnerable to

disease and other unforeseen evolutionary pressures. It has been suggested that the lack of genetic variation in Spoonbills may account for the observed high (50%) fledgling mortality rate (Rebecca Linn, 1993) and an overall vulnerability of both wild and captive populations to potentially catastrophic disease outbreaks.

Spoonbills have been bred and maintained at the Fort Worth, Dallas and other zoos for several decades. However, there is a general lack of pedigree information on these captive populations. Familial relationships of Spoonbills are very difficult to establish by traditional observation techniques. This is because males often share nests with different females, and females sometimes lay their eggs in nests to be raised by other Spoonbill pairs (Chris Brown, personal communication). The possible genetic effects of inbreeding within the captive populations, and the previous impact of the population bottleneck on the wild population from which these were drawn, are not known, but if substantial may lead to the expression of genetic defects or weaknesses. This has already occurred in highly inbred strains of chickens and would hinder some aspects of a progressive captive Spoonbill breeding program.

The rapid advances in molecular genetics of the last decade have led to an accelerating application of this area of biotechnology to numerous animal breeding programs. The development of DNA-based breeding programs for zoos has made the maintenance of genetic diversity much easier in captive populations of large carnivores such as cats (in particular Cheetahs-*Acinonys jubatus*) and large primates. Applying DNA-based technology to breeding programs for captive bred avian species can be expected to be equally useful, indeed necessary, in this age of increased conservation

awareness and wildlife preservation under the auspices of the Wild Bird Conservation Act (1992).

DNA-based identity tests

The creation of a DNA-based identity test for a species is dependent upon isolating and characterizing loci which exhibit a high level of polymorphism and heterozygosity in the population to be studied. Such loci are relatively common in higher eukaryotes and have been found, for example, in many mammalian genomes (human, mouse, whale, etc.) as well as in numerous avian genomes (chickens, pheasant, finches, etc.). Modern techniques utilized for the purpose of DNA-typing individuals of a population with polymorphic loci include: (1) nucleotide sequence determination of hypervariable regions, such as the "control region" of mitochondrial DNA (mtDNA) (Allen *et al.*, 1998), (2) restriction fragment length polymorphism (RFLP) analyses (with single- and multi-locus probes), and (3) polymerase chain reaction (PCR) (Saiki *et al.*, 1988) analyses of polymorphic short tandem repeat (STR) and Major Histocompatibility Complex (MHC) loci.

Previous Spoonbill research

Initial attempts to DNA-type Spoonbills using known multilocus mammalian molecular markers and probes provided limited useful data (Linn, 1993). RFLP analyses of mtDNA allowed some identification of breeding females. However, inbreeding of offspring in future years resulted in a rapid loss of polymorphism at these loci. This is not a surprise since the loci testable by this technique was limited to the few polymorphic restriction endonuclease cutting sites within the bird's 15-17 kbp mtDNA chromosome

(Brown *et al*., 1979). Linn also tried DNA RFLP profiling (Jeffreys *et al*., 1985a) of *Hae*III- and *Hin*fI-cut Spoonbill DNA with the M13-derived minisatellite probe (Devor *et al.*, 1988) and screened the Spoonbill genome for $(GT)_n$ microsatellites. Only the M13 probe with *Hae*III-cut DNA yielded a polymorphic profile (two alleles, 3 possible haplotypes). The microsatellite frequency in Spoonbills was determined to be extremely low, approximately 1 per 1.6-4.0 mb versus 1 per 30-40 kb in humans (Weber and May, 1989; Linn, 1993). Linn's experimental studies indicated a "severely limited level of genetic variation (polymorphism) in the Fort Worth and Dallas Roseate Spoonbill colonies." Additionally, "the parentage of most of the birds could not be identified".

Mitochondrial DNA analysis

Future studies using Spoonbill mtDNA may include complete DNA sequence determination of the control region (D-loop), which has been shown to be hypervariable in many organisms (Quinn and Wilson, 1993). Analysis of the mtDNA control region at the nucleotide level is more powerful than the simpler RFLP studies performed by Linn (1993), which require sequence differences to occur only at restriction sites in order for them to be detected. By determining the exact DNA sequence of individual Spoonbill control regions, single nucleotide polymorphisms (SNPs) can be identified and estimates of relationships may be calculated. Offspring from a female should have identical mtDNA sequences as their mother, unless a random mutation has occurred, since mtDNA is maternally inherited (Lansman *et al*., 1983). Such a mutation would still allow a very good estimate of familial relationships, which could be confirmed by more powerful DNA identity tests.

Restriction Fragment Length Polymorphism analysis

The most commonly used, and powerful, identity tests are RFLP analyses with single-locus probes for variable number tandem repeats (VNTRs) and PCR amplification of polymorphic STR loci (Corach *et al*., 1995; Delahunty *et al*., 1996). Each of these is currently used to DNA type humans for paternity and forensic investigations. Other less powerful DNA identity tests include multi-locus probing (Jeffreys *et al*., 1985b) of restriction digested DNAs (RFLP analysis) and random amplified polymorphic DNA (RAPD) analysis via PCR (Nusser *et al*., 1996). These latter tests rely on the analysis of multiple band patterns obtained either by autoradiography (multi-locus probing) or by PCR amplification and electrophoretic analysis of the DNA products. Multiple band patterns from multilocus probes require complex statistical analyses in order to make estimates of uniqueness or relatedness of individuals and are generally less useful.

Major Histocompatibility Complex of birds

The discovery and isolation of polymorphic loci in a new species may be accomplished by examining areas of the genome, such as the MHC, which are known to be highly polymorphic in previously characterized, and related, species (Zoorob *et al*., 1993). The chicken MHC (*B*-complex), originally described by Briles *et al*. (1950) as a blood group locus, has become the most extensively characterized non-mammalian MHC (Kroemer *et al*., 1990). We now know that the chicken MHC is simpler and smaller than the mammalian, human and mouse MHC (Kaufman and Wallny, 1996). Most of the chicken MHC exists on chromosome 16 as the *B-F/B-L* region. It determines rapid

allograft recognition, and the *B-G* region, encoding the erythrocyte antigens, is generally used for serological typing. Some chicken MHC genes encode polymorphic *B-F* and *B-L* cell surface antigens functionally equivalent to mammalian class I and II molecules, respectively (Guillemot *et al*., 1988; Lamont, 1993). Another chicken locus, *Rfp-Y*, which contains four MHC loci and two genes, is also present on chromosome 16 (Miller *et al*., 1996). The extensive knowledge base concerning the chicken MHC region, the availability of chicken MHC genomic DNA probes, and the high level of sequence conservation of the MHC between species (Hashimoto *et al*., 1990), make this region a promising choice for DNA-based identity test development in Spoonbills.

Isolation of Short Tandem Repeats

The highly polymorphic STR loci, also known as microsatellites, have been found to occur extensively throughout the genome in higher eukaryotes and usually consist of di-, tri-, or tetranucleotides units repeated tandemly numerous times. The construction of eukaryotic (human, mouse, bovine and chicken) genetic maps over the last decade has been greatly facilitated by the characterization of polymorphic microsatellite loci within each species. Microsatellite loci can be quickly analyzed for allele identification by PCR amplification, once the locus has been sequenced and proper PCR primers designed. Linkage analysis can then be used to generate sequence tagged site (STS) gene maps (Steffen *et al*., 1993). Isolation of Spoonbill STR loci, and determination of unique flanking region nucleotide sequences, would allow for the design of locus-specific primers for PCR amplification of these sites and facilitate the quick evaluation of multiple loci for allele identification. Since these loci are inherited in a Mendelian

manner, each individual is expected to have one (homozygous) or two (heterozygous) bands, dependent upon which alleles their parents passed on. The disadvantage of mitochondrial typing, maternal inheritance of only one chromosome, is overcome and loci can be used from each syntenic group independently.

There are several published methods designed to enrich genomic DNA samples for fragments encoding STRs and their reported yield efficiency has ranged from 0.3% to 35% (Rafseth *et al*., 1997). Factors such as the frequency and repeat length of STRs in a species may affect the success of particular method. The microsatellite frequency in chickens is estimated to be about tenfold less than that found in mammals (Crooijmans *et al*., 1993) and Linn's study has already determined the frequency to be considerably lower (1993). Although the STR frequency in Spoonbills may be slightly different from that in chickens, since phenetically and phylogenetically they belong to separate orders (Stevens, 1996), an assumption of lower frequency compared to mammals is valid. Therefore, the use of the most efficient method(s) of STR isolation is preferable to ensure capture of STR regions.

Studies have been performed to enumerate the most and least frequent types of microsatellite loci within avian genomes (Longmire *et al.*, 1999; Primmer *et al.*, 1997). Many of the STRs with the highest frequencies within avian genomes were chosen for this study (Table I, page 61). Although dinucleotide repeats are more numerous (Tautz *et al*., 1986; Shriver *et al*., 1993), and therefore easier to find (Mindell, 1997), they are more difficult to genotype because of multiple anomalous bands which appear during the scoring process, resulting from slippage during PCR amplification. Interestingly, the current theory used to explain the mutation mechanism by which the hypervariable

microsatellite occurs, involves the process of "strand slippage" during DNA replication (Schlötterer and Tautz, 1992).

Magnetics-based STR capture

An innovative bioseparation technique previously used primarily for mRNA purification was modified to isolate STRs from genomic DNA (Gardner *et al*., 1999). This technique is magnetics-based, utilizing streptavidin-coated paramagnetic particles (SA-PMPs) and a magnetic separation stand to bind biotin-labeled oligonucleotides which were specifically made to complement an STR of interest (Fig. 1). Hybridization of size-fractionated genomic DNA (300-800 bp) to the biotinylated oligonucleotides, which are attached to SA-PMPs, allows capture of STR-rich genomic DNA. Genomic DNA not containing STRs, and which does not hybridize, is washed from the mixture. The captured genomic fragments are then eluted from the biotinylated oligonucleotides, immediately amplified by a PCR amplification to create double-stranded products, and then cloned into a vector to create an STR-enriched genomic library. The STR-enriched library may then be screened for specific clones containing the STR of interest. Screening may proceed with the transformation of *E. coli*, followed by the DNA sequence analysis of each transformant. Alternatively, the screening may be performed faster by the PCR amplification of each colony with 3 primers. Two primers would bind to the flanks of the multiple cloning site and one would bind internally to complement the STR of interest (Gardner *et al*., 1999). If the STR is present within the clone, two bands would be produced, one relative to the size of the entire insert and the other the result of the amplification between the STR locus and a flanking primer.

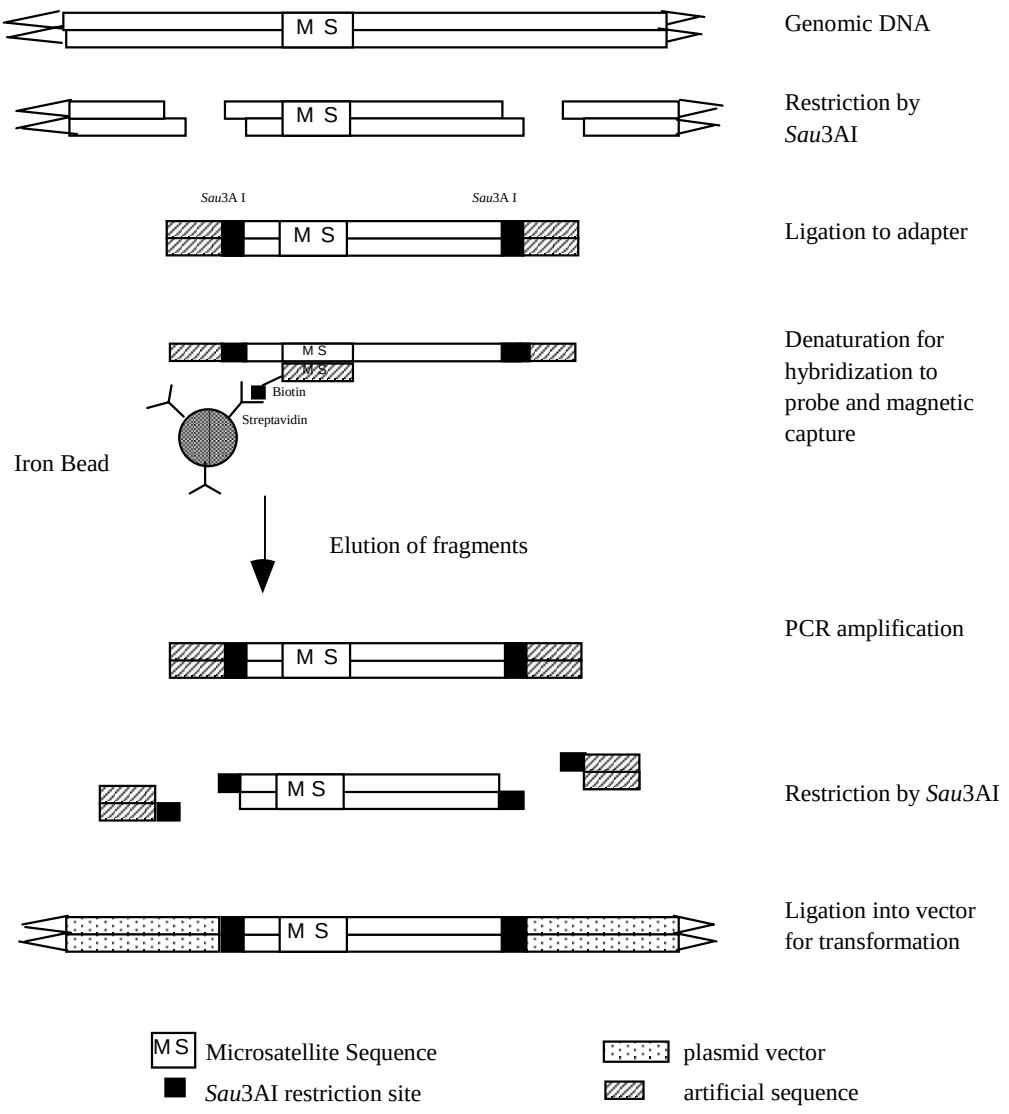

Figure 1. **Flow chart of magnetics-assisted microsatellite capture**. The short tandem repeat (STR)/microsatellite enrichment procedure utilized biotinylated probes and streptavidin-coated beads for capture of microsatellite sequence fragments from the Spoonbill genome (schematic adapted from Fischer and Bachmann, 1998).

Research objectives

This study was an attempt to isolate a minimum of five polymorphic loci which could be used to identify each of the 51 Spoonbills. Oligonucleotide primers would be designed for each potential polymorphic locus to allow PCR amplification of genomic DNA samples. Any size differences between the amplification products would be quantified, relative to a size standard, to enable comparisons of all samples at the same locus. It was preferable to obtain loci with high frequencies of observed heterozygosity (H_O>0.5) and allelic diversity, which would conclusively identify members of the Zoo populations. Since the availability of more loci would increase the power of identification, as many loci as possible were to be obtained. The allele(s) from each individual's polymorphic locus would determine its allelic profile for that specific locus. The collection of allelic profiles would constitute the DNA profile or "fingerprint" of an individual, to be used in lineage determination (where applicable) among the Zoo populations, or at least the assignment of a genetic profile, which could be accessed through a database. If necessary, DNA sequence differences in the mtDNA control region would be analyzed and used to help elucidate any questionable lineage.

In an effort to understand the degree of genetic diversity within the wild population, Spoonbills were to be collected from the wild. When attempts to obtain the necessary permits and personnel could not be finalized, Spoonbills known to have originated from the wild were collected from the Zoo populations, which created four populations for analysis purposes; Dallas Zoo, Fort Worth Zoo, Sedgwick County Zoo, and the wild population. By comparing the DNA profiles of individuals from the wild and captive populations, both within and between the groups, an understanding of the

degree of genetic differences was to become apparent, which should greatly assist in long term species management decisions.

The research objectives of this project are to develop a set of DNA-based tests which could be used to establish a genetic profile for individual Roseate Spoonbills and allow the determination of lineage within captive populations. To accomplish the objectives of this project the following aspects must be completed:

1. Identify loci exhibiting sequence polymorphism among the study populations.
2. Develop PCR-based identity tests.
3. Identify the alleles present at each locus by their size, estimate the frequency of each allele in the total population (51 Spoonbills), and compare the observed and expected rates of heterozygosity.
4. Identify the alleles of each Spoonbill and determine if the reported lineages from the Zoos are accurate and, where possible, assign parentage of offspring.
5. Apply published and newly discovered (this project) sex-specific PCR tests to individual Spoonbills to verify or identify their sex.

The application of these identity tests on individual Spoonbill DNA samples, even when there are no known relationships, should allow assignment of alleles to a database from which all Spoonbills can be compared. Each Spoonbill is represented by a numerical list of specific alleles which constitutes its genetic profile. From a collection of these profiles, a population manager is better informed as to the potential alleles available from a particular Spoonbill and can decide which Spoonbill will better assist the future diversity of the population.

CHAPTER II

MATERIALS and METHODS

Bacterial strains and plasmids

Escherichia coli DH5α™ (*supE44, hsdr17, recA1, endA1, gyrA96, thi-1* and *relA1*) (Sambrook *et al*., 1989) was the bacterial host strain used to propagate the clones created within the vectors pUC18/19 and pBluecript® II SK(-), each of which contains the ColE1 origin of replication.

E. coli XL1-Blue MRA [Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 gyrA96 relA1 lac*] was the bacterial host strain used for the growth of cloned fragments propagated in the Lambda DASH® II replacement vector (Stratagene®, 1997a).

E. coli XL1-Blue MRA (P2) (P2 lysogen of XL1-Blue MRA) was used as the recipient strain for recombinant Lambda DASH® II clones when it was necessary to select against the growth of wild-type Lambda DASH® II phage (Stratagene®, 1997a).

E. coli XL1-Blue MRF' [Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac {F' proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})}] was the host strain used to propagate lambda ZAP Express® clones (Stratagene®, 1999).

E. coli XLOLR [Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 thi-1 recA1 gyrA96 relA1 lac {F' proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^{r})} Su^{-} (nonsuppressing) λ^{r} (lambda resistant)] was the host strain for plating excised phagemids. The pBK-CMV phagemid clones are created/derived from the lambda ZAP Express® clones through the use of the

XLOLR strain with the help of ExAssist® interference-resistant helper phage (Stratagene®, 1999).

Biological media and growth conditions

Media used for this study included LB and NZY broth. The addition of 1.5% agar to either of these liquid media produced solid media. DH5α™ was cultivated in LB (Luria-Bertani) medium consisting of 1.0% (w/v) Bacto Tryptone™, 0.5% (w/v) yeast extract and 1.0% (w/v) sodium chloride. Whenever DH5α™ carried an ampicillin-resistant plasmid or clone, ampicillin (50 µg/ml final concentration) was added to the LB medium. When DH5α™ carried a kanamycin-resistant plasmid, kanamycin (50 µg/ml final concentration) was added to the LB medium. MRA and MRA (P2) were cultivated in LB or NZY broth supplemented with 0.2% (w/v) maltose-10 mM $MgSO_4$ in order to optimize lambda phage attachment to the cell membranes. NZY broth contained 0.5% (w/v) NaCl, 0.2% (w/v) Mg SO_4, 0.5% (w/v) yeast extract, and 1% (w/v) NZ amine (casein hydrolysate). NZY Top Agar was made by the addition of 0.7% (w/v) agarose to NZY medium.

The XL1-Blue MRF' and XLOLR strains were maintained on LB-tetracycline plates. Tetracycline was added to the LB agar medium at 12.5 µg/ml final concentration. Maltose at 0.2% (w/v) final concentration and magnesium sulfate ($MgSO_4$) at 10 mM final concentration were added to the LB or NZY (both without tetracycline) for titering lambda phage using XL1-Blue MRF'. The XLOLR strain was cultured in NZY medium without the use of supplements.

Long-term storage of cultures

Bacterial strains were stored at –75°C. Prior to storage each strain was grown overnight in a shaker incubator at 37°C and 250 rpm in a 5 mL LB broth tube. If plasmids were present in the strain, the antibiotic ampicillin (at 50 µg/ml final concentration) was used during the overnight incubation to maintain the selection for the plasmid. Each overnight growth was converted into a 40% glycerol stock by the mixing 500 µl of sterile 80% glycerol with 500 µl of the overnight culture. After a thorough mixing by repeated inversion, the tubes were placed in an ethanol bath at –50°C for quick freezing, then at –75°C for long term storage.

Genomic DNA isolation from avian whole blood

Most of the circulating blood cells in birds and reptiles are nucleated, in contrast with mammals where only the leukocytes and immature erythrocytes contain nuclei. This physiological difference was realized in a practical experiment when a 5 µl sample of avian whole blood yielded approximately the same amount of DNA as that from 5 ml of mammalian whole blood (200-500 µg). In order to isolate an amount of DNA which could be processed in a standard 1.5 ml microcentrifuge tube, 5 µl of whole blood from each Spoonbill was placed into a sterilized 1.5 ml microcentrifuge tube. Fifty microliters of cold Lysis Buffer (0.32 M sucrose, 5 mM magnesium chloride, 1% Triton X-100 and 10 mM Tris-HCl, pH 7.5) was added to each tube and inverted repeatedly to mix well. After the samples were left on ice 20 minutes they were placed into a chilled (4°C) Savant™ HSC10K and centrifuged at 2,800 x g for 10 minutes. The supernatant was removed and 50 µl of cold lysis buffer was added followed by brief vortexing to disrupt

and resuspend the pellets. The suspensions were left on ice for 5 minutes and then centrifuged again at 2,800 x g for 10 minutes. After the supernatant was discarded, 400 µl of Digestion Buffer (750 mM NaCl, 240 mM Na_2EDTA) was added and each tube was vortexed very well. Twenty microliters of 20% SDS and 16 µl of Proteinase K (10 mg/ml) were added to release the DNA from its compact chromatin state of being wrapped in histones and associated with the nuclear matrix. The tubes were inverted back and forth gently to mix well and allowed to incubate for 1 hour at 50°C. Further incubation at 50°C was used if necessary to digest any large debris. One hundred microliters of 6 M $NaClO_4$ (sodium perchlorate) was added to stop the activity of Proteinase K as well as add salt to the aqueous solution. The tubes were mixed well by gentle inversion for 10 minutes.

A phenol/chloroform extraction procedure was then performed to remove any proteins and lipids. First, 600 µl of TE-saturated phenol was added to each sample, which was then inverted repeatedly to mix. Centrifugation at 10,000 x g for 2 minutes was performed in a room temperature Savant™ HSC10K. Ninety percent of the phenol was removed by a mechanical pipetor and 600 µl of Extraction Reagent (50% phenol: 49% chloroform: 1% isoamyl alcohol) was added. The tubes were mixed well by quick inversion and then centrifuged at 10,000 x g for 2 minutes. Each supernatant was moved to a new, sterile 1.5 ml microcentrifuge tube. Six hundred microliters of chloroform/isoamyl alcohol (24:1) was added to each sample. These were mixed well by repeated inversion and then centrifuged at 10,000 x g for 2 minutes. The supernatants were again transferred to a new, sterile 1.5 ml microcentrifuge tube. Precipitation of the DNA from each sample was begun by the addition of 66 µl 3 M sodium acetate and 1 ml

ice-cold 100% ethanol. The samples could then be mixed well by invertion and placed in a dry ice/ethanol bath for 2 minutes. After centrifugation at 10,000 x g for 10 minutes at 4°C, the supernatant could be removed carefully by use of a drawn-out Pasteur™ pipet. The samples were washed by the addition of 1 ml ice-cold 70% ethanol, inverting once, and centrifugation at 10,000 x g for 5 minutes at 4°C. The supernatant was removed with a drawn-out Pasteur™ pipet and the tubes were placed into a Savant Speed Vac™ vacuum concentrator for 2-3 minutes to remove any residual ethanol. Each DNA sample was resuspended in 100 µl ddH_20. In order to determine the DNA concentration of each sample, a dilution of 1:100 (10 µl sample into 990 µl ddH_2O) was analyzed by a Beckman™ DU®-40 spectrophotometer (see Quantitative and qualitative identification of DNA). A typical yield from 5 µl of blood was in the range of 20 µg.

Quantitative and qualitative characterization of DNA samples

The quantitation (concentration determination) of nucleic acids was paramount for optimizing the DNA to enzyme ratios utilized in many procedures. When synthetic oligonucleotides were received from the manufacturer (generously provided by Bio-Synthesis, Inc.) they were diluted and assayed with a Beckman™ DU®-40 spectrophotometer. This procedure became less necessary because of the advent of higher quality oligonucleotide synthesizers. However, machines and/or operators of oligo-synthesizers have made mistakes in the past. Therefore, a cursory analysis to determine yield and quality of the newly acquired oligonucleotide was routinely performed. First the oligonucleotide was reconstituted from its desiccated form in 330 µl ddH_20 (5 OD units), and then was diluted 1:100 by placing 10 µl oligonucleotide solution

into 990 µl ddH_20 for spectrophotometer analysis. The diluted DNA sample was then placed into a 1 ml quartz cuvette for a wavelength scan from 220 nm to 320 nm. An absorbance reading of 1.0 in the range of 256–260 nm represented approximately 33 µg of single-stranded DNA per ml. Double-stranded DNA was also routinely analyzed where an absorbance of 1.0 (at 256–260 nm) represented approximately 50 µg of double-stranded DNA per ml. Phenol or protein contaminants, if present, would shift the absorbance maximum to the right (270 nm-280 nm).

Nucleic acids were also evaluated by staining with the intercalating agent ethidium bromide (EtBr) following gel electrophoresis. EtBr emits an orange fluorescence when exposed to UV irradiation. The EtBr intercalates between the base pairs of DNA and allows the detection of DNA by use of a UV transilluminator or a hand-held UV source. Marker DNA of known size and concentration was loaded adjacent to experimental DNA samples and following electrophoresis the gel was stained with EtBr solution (0.5 µg/ml). A visual comparison of the experimental samples to the marker samples allowed for estimations of size and concentration. The quality of experimental nucleic acid samples was estimated by the shape, size and location of stained material on EtBr-treated gels.

Restriction endonuclease digestion of DNA

The cleavage of DNA at specific palindromic sites by restriction endonucleases (restriction enzymes) was essential in creating clones for libraries, characterization of large clones or fragments by restriction mapping, or even creating specific ends on polymerase chain reaction (PCR) products, so that they may be cloned into vector DNA

with compatible ends. All restriction enzymes and their appropriate buffers were purchased from New England Biolabs. Each restriction endonuclease was used at its optimum temperature of 25, 37 or 65°C for 2 to 4 hours. The enzymatic reactions were designed to have 2 to 10 units of enzyme per µg of duplex DNA, as long as the enzyme volume never exceeded 10% of the total reaction volume. Enzyme storage buffer consists of 50% glycerol, which allows the enzyme to be stored at –20°C for long periods (years). The 10% limit of enzyme in a reaction mixture maintained a lower glycerol level and helped prevent altered specificity of the restriction endonuclease. A standard restriction enzyme digestion was set up as follows:

1 µl	restriction enzyme (5 U/ µl)
2 µl	10X reaction buffer (provided by the manufacturer)
6 µl	DNA (0.5 µg/ µl)
11 µl	sterile distilled deionized water
20 µl	Total volume

Many enzymes were inactivated by heat (65°C for 20 minutes) in order to ensure no further cutting may occur during a ligation reaction. Alternatively, the restriction enzyme digestion was inactivated by phenol extraction and ethanol precipitation (see Phenol extraction of DNA solutions) which removed any enzymes and salts present.

Alkaline phosphatase treatment of vector DNA

The removal of the 5' phosphate from nucleic acids was extremely useful in limiting the number of self-ligations of vectors during cloning strategies. The calf intestinal phosphatase (CIP) produced by New England Biolabs was used since it was

active in most of the restriction enzyme buffers produced by New England Biolabs. Only additional diluted buffer and CIP needed to be added to a restriction digestion in order to remove the 5' phosphoryl group. The typical reaction mixture was as follows:

20 µl	restriction digestion (linear DNA)
35 µl	ddH_20
4 µl	10X reaction buffer (New England Biolabs)
1 µl	calf intestinal phosphatase (10 U) (New England Biolabs)
60 µl	Total volume

The reaction was placed at 37°C for 1 hour. The sample of phosphatase-treated linear vector DNA could then be phenol extracted to inactivate the phosphatase and precipitated with ethanol to remove the buffer salts. Usually each phosphatase-treated vector was resuspended in 60 µl ddH_20 for a final concentration of 50 ng/µl.

Phenol extraction of DNA solutions

The removal of enzymes from a restriction digestion, ligation or phosphatase treatment was often begun with the organic solvent phenol. Phenol was used to denature proteins, which allowed them to precipitate from an aqueous solution and be removed from the DNA. A high quality (distilled) phenol stock was prepared for DNA solution cleanup by saturating it with TE buffer and the addition of 0.1% (w/v) 8-hydroxyquinoline. The 8-hydroxyquinoline was used to color the phenol yellow (to distinguish it from water) and to act as an anti-oxidant.

TE buffer or water was added to a tube containing an enzymatically-treated DNA solution to increase the volume to 100 µl. An equal volume (100 µl) of TE-saturated

phenol was then added and mixed well by vortexing. The tube was then centrifuged for 3 minutes at 10,000 x g. The lower layer of phenol was removed using a micropipettor and an additional 100 µl of phenol was added. The sample was vortexed again, centrifuged and the phenol removed. To facilitate the complete removal of phenol, 500 µl diethyl ether was added, the sample vortexed well and centrifuged at 10,000 x g for 15 seconds. The ether layer (top) was removed with a drawn-out Pasteur™ pipet. Any residual ether was allowed to volatilize by placing the tube in a vacuum exhaust hood for 5 minutes at room temperature with the lid open. To precipitate the DNA, 11 µl of 3.0 M sodium acetate and 350 µl of –20°C 100% ethanol were added and mixed by vortexing. The tube was then centrifuged in a chilled (4°C) microcentrifuge at 10,000 x g for 10 minutes, followed by the removal of the supernatant with a drawn-out Pasteur™ pipet. In order to remove any residual salts in the tube, 500 µl of –20°C 70% ethanol was added. The tube was inverted gently before centrifuging in a chilled microcentrifuge at 10,000 x g for 5 minutes. The supernatant was removed with a drawn-out Pasteur™ pipet and placed open in a Savant Speed Vac™ vacuum concentrator for 5 minutes. The dried DNA pellet was then resuspended with sterile ddH_20, or TE buffer, to a useful concentration. The DNA solution was stored at –20°C until use.

Horizontal submarine agarose gel electrophoresis

Agarose gel electrophoresis allows a researcher to both quantify and qualify nucleic acids. For PCR-amplified products of less than 600 bp, a 2% agarose solution was used, which allowed slightly better resolution than the 1% gels typically used to analyze larger plasmid samples. An agarose gel form was created using masking tape to

seal the ends of an acrylic gel tray. A comb containing the necessary amount of teeth was placed at one end of the tray at a perpendicular angle. The agarose gel solution was then prepared by heating the tared combination of agarose and 1X TBE (5.4 g Tris base, 2.75 g boric acid, 2.0 ml 0.5 M EDTA, pH 8.0 in 1 liter ddH_2O) in a microwave, which was subsequently re-balanced with deionized water to maintain the correct agarose percentage. The molten solution was then poured into the tray until the teeth of the comb were approximately 70% covered. When the gel had sufficiently cooled to produce a solid slab (45 minutes), the comb was carefully removed and the gel was placed in the electrophoresis chamber with the wells at the negative electrode end. The chamber was then filled with 1X TBE such that only 1-2 mm of buffer covered the gel. Each DNA sample was mixed with an appropriate amount of 5X loading buffer (25% glycerol, 0.5% SDS, 0.1% bromophenol blue, 0.1% xylene cyanol FF, 50 mM EDTA) and placed into a well. Electrophoresis was performed at 4-6 V/cm (e.g. 80 V in a 17 cm-long chamber) for 1.5 hours or until the desired distance of migration was attained. The gel was then placed in an EtBr staining solution (0.5 μg/ml in ddH_2O) for 15 minutes, followed by 10 minutes in distilled water to remove the excess EtBr. The gel was then transferred to a UV transilluminator and the DNA fluoresced under UV irradiation while being digitally photographed with the K*odak* D*igital* S*cience*™ Electrophoresis Documentation and Analysis System 120, Limited Edition (Kodak™ EDAS 120, LE).

Ligation of DNA fragments into cloning vectors

The 5' ends of target DNA molecules were joined to complementary ends of restriction enzyme digested vector DNA by use of the enzyme T4 DNA ligase (GIBCO

BRL®). The formation of these recombinant DNA molecules, colloquially known as clones, was optimally performed in a ligation reaction consisting of a one-to-one molar ratio of vector to target DNA. Usually 100 ng of dephosphorylated vector DNA was combined with various quantities of target DNA in order to arrive at the one to one ratio. If the desired product was not successfully cloned, the molar ratio was adjusted as needed, incrementally, from 10/1 to 1/10 vector/target. The ligation buffer supplied by the manufacturer (GIBCO BRL®) was used for 6-8 weeks or until the ligation efficiency decreased. At this time either new ligation buffer was purchased from the manufacturer or created in the laboratory. The 5X ligation buffer consisted of 250 mM Tris-HCl, pH 7.6, w/v PEG 8000 (polyethylene glycol, av. mol. wt. 8000), 50 mM $MgCl_2$, 5.0 mM ATP, and 5.0 mM DTT. Four microliters of 5X ligation buffer and 1 µl T4 DNA ligase (1 U) were used in a standard 20 µl reaction volume. Target and vector DNAs in sterile ddH_2O were added to complete the reaction mixture. The ligation proceeded at room temperature for a minimum of 3 hours for complementary-ended ("sticky-ended") substrates or at 16°C overnight (16-20 hours) for blunt-ended substrates. Ligated plasmid DNAs were then transformed into DH5α™, freshly made competent by the TSS method, or into competent cells stored at -80°C in 200 µl aliquots.

Recombinant plasmid DNAs were created in great quantities during the formation of a size-selected genomic DNA library. Spoonbill genomic (target) DNA was digested with a restriction endonuclease and ligated into a compatible restriction site on a plasmid vector which had been de-phosphorylated at its 5' termini. Therefore, the 5'-ends of the target DNAs were instrumental in formation of stable phosphodiester linkages with both ends of a restriction-digested vector. Whenever a recombinant clone was isolated, the

DNA sequence was determined using primers which were specific to the known regions flanking the multiple cloning site (MCS).

Preparation of competent *E. coli* DH5α™ cells for transformation

E. coli DH5α™ were treated chemically to prepare them for the transformation process. The ColE1-derived plasmids pUC18 and pBluescript® II SK(-) were routinely used as cloning vectors and subsequently transformed into chemically competent DH5α™. A 16 mm x 125 mm culture tube with 5 ml LB broth was inoculated with a single colony of DH5α™ from a fresh LB agar plate. This culture was incubated at 37°C overnight in a New Brunswick™ Series 25 gyratory incubator shaker at 250 rpm. Five hundred microliters of the overnight growth was then aseptically transferred to 50 ml LB in a 250 ml Erlenmeyer flask. This was placed in the shaker incubator at 37°C and 250 rpm. After 2 hours, a 1 ml aliquot from the culture was transferred to a plastic cuvette and analyzed with a Beckman™ DU®-40 spectrophotometer. Once the OD_{550} of the culture reached 0.45, it was transferred to a sterile 50 ml polypropylene tube and placed in an ice water bath for 10 minutes. The tube was then centrifuged at 1,000 x g for 10 minutes at 4°C. The supernatant was poured off and the cellular pellet was resuspended by "brief vortexes" in 4 ml TSS solution (85% LB broth, 10% w/v PEG 8000, 5% v/v dimethylsulfoxide, and 50 mM $MgCl_2$, pH 6.5). The resuspended cells were kept in a slurry of ice water until ready for use. If the cells were not to be used within 6 hours, 200 µl aliquots were transferred to 1.5 ml microcentrifuge tubes and quickly frozen in a –50°C ethanol bath. The tubes would then be transferred to a –80°C freezer and were

thawed on ice when needed. The cells were generally viable and competent for up to 6 months.

Transformation of *E. coli* DH5α™ with recombinant pUC18/19 and pBluecript® II SK (-) plasmids

E. coli DH5α™ was the strain used for transformations of all recombinant ColE1 (pUC and pBluecript®)-derived plasmids. Four nanograms (ng) of purified plasmid or 50-100 ng from a ligation reaction was added to 200 µl competent cells in a 1.5 ml microcentrifuge tube. The tube containing the DNA and cells was gently mixed and placed into an ice water bath for a minimum of 10 minutes. A heat shock procedure was then performed by transferring the tube to a 42°C water bath for 2 minutes. A 1 ml aliquot of sterile LB medium was added to the tube, which was subsequently inserted into the top of an empty culture tube and placed into a gyratory incubator and shaker at 37°C for 45 minutes at 250 rpm. This incubation period allowed for the out-growth, or expression, of the ampicillin resistance gene (*bla* gene: β-lactamase) carried by ColE1-derived plasmids such as pUC18/19 and pBluescript®. Selection for transformed bacteria was accomplished by spread-plating 30 and 200 µl aliquots of the microcentrifuge tube contents onto separate LB agar plates containing ampicillin (50 µg/ml), which were pre-spread with 10 µl 100 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 50 µl of 2% 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-Gal). Each plate of cells was incubated at 37°C for 12 to 20 hours. The only cells which could grow on the antibiotic-containing plates were ones that had been transformed by a plasmid containing the antibiotic

resistance gene. If the plasmid were non-recombinant (no additional DNA fragment inserted into the MCS), it would produce the LacZ α-peptide necessary to complement the *lacZ*ΔM15 gene of the host cell and thereby create a functional β-galactosidase. This cleaves X-Gal to yield a blue color observable in the growing colonies. A recombinant plasmid has a DNA fragment inserted into the LacZ α-peptide gene at the MCS, rendering the gene inactive. Therefore the recombinant bacterial colonies would have the phenotypic appearance of white or opaque.

Rapid plasmid isolation using an alkaline lysis technique

This technique was adapted from Birnboim and Doly, 1979. Bacterial cells either from an isolated colony on a transformation plate or from a master plate streak were inoculated into a culture tube containing 5 ml of LB broth supplemented with 50 μg ampicillin per ml. The tube was then placed in a shaker incubator at 37°C and 250 rpm to incubate for 12 to 24 hours. A 1.5 ml aliquot of the culture was transferred into a sterile 1.5 ml microcentrifuge tube and the tube was centrifuged at 10,000 x g for 1 minute. The supernatant was poured off and an additional 1.5 ml aliquot of the same culture was added to the microcentrifuge tube. The tube was centifuged at 10,000 x g for 1 minute. Most of the supernatant was removed by aspiration with a drawn-out Pasteur™ pipet. The bacterial cell wall was then lysed by the addition of 100 μl ice-cold Solution A (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0, 6 mg/ml lysozyme). The sample was vortexed very well and incubated at room temperature for 5 minutes. Two hundred microliters of freshly prepared Solution B (0.2 N NaOH, 1.0% SDS) was then added and mixed by inversion 3-4 times. This was followed by a 5 minute incubation on

ice. One hundred fifty microliters of ice-cold potassium acetate (approximately pH 4.8) was added to the tube and the contents were mixed by inverting 3-4 times. The tube was stored on ice for 5 minutes followed by a 4°C centrifugation at 10,000 x g for 5 minutes. The supernatant was transferred to a new sterile microcentrifuge tube to which an equal volume (450 µl) of phenol:chloroform (1:1) was added. The sample was mixed well by vortexing and centrifuged for 2 minutes at 10,000 x g to create two layers, the upper aqueous and the lower organic. The upper aqueous layer was then transferred to a new sterile microcentrifuge tube and 750 µl of -20˚C 100% ethanol was added. The contents of the tubes were mixed well by vortexing and centrifuged at 4°C for 10 minutes at 10,000 x g. The supernatant was removed with a drawn-out Pasteur™ pipet and 1 ml of -20˚C 70% ethanol was added. The tube was inverted once, centrifuged at 4°C for 5 minutes at 10,000 x g, and the supernatant removed by aspiration. The DNA pellet was dried for two minutes in a vacuum desiccator to remove any residual ethanol. Forty microliters of TE buffer (pH 8.0) with heat-treated RNase A at 25 µg/ml was added to the tube and vortexed well to dissolve the DNA. Characterization of the DNA could then be performed by electrophoresis, restriction endonuclease digestion, and/or DNA sequence analysis.

Preparation of radio-labeled probes

The detection of nucleic acid sequences present on nylon membranes, via Colony/Plaque Screen™ membranes (lifts) or Southern (1975) blotting, was performed by hybridization with radiolabeled probes followed by autoradiography. Three different

types of radiolabeled probes were created for this study; 5' end-labeled oligonucleotides, 3' end-labeled oligonucleotides, or random-primed DNA templates.

The production of 5' end-labeled oligonucleotides was performed by the incorporation of γ-labeled ^{32}P dCTP onto the 5' position of the terminal nucleoside. A 5' end-labeled probe was prepared by mixing the following together in a microcentrifuge tube.

2 µl	oligonucleotide (5 pmol/µl)
2 µl	10X polynucleotide kinase (PNK) buffer (New England Biolabs)
5 µl	γ-labeled ^{32}P dCTP @6000 Ci/mmol
10 µl	ddH_20
1 µl	T4 polynucleotide kinase (10 U/µl) (New England Biolabs)
20 µl	total volume

This mixture was incubated at 37°C for 1 hour. Each probe was purified from the unincorporated nucleotides by exclusion chromotograhpy using Sephadex™ G-25 matrix (see Spun-column chromatography for probe or large-fragment cleanup).

The production of 3' end-labeled oligonucleotides was performed by the incorporation of α-labeled ^{32}P (dATP) onto the 3' position of the terminal nucleoside. Since the 3' end incorporates a deoxynucleotide, multiple α-labeled ^{32}P nucleotides may be incorporated, by extending the DNA molecule in the 3' direction, resulting in an increased specific activity of the radiolabeled oligonucleotide relative to one deoxy-nucleotide incorporated via the 5' end-labeled method. A 3' end-labeled probe was prepared by mixing the following together in a microcentrifuge tube.

20 µl	STR-3 oligonucleotide (0.5 pmol/µl)

10 µl 5X terminal deoxytransferase (TdT) buffer (Stratagene®)

2 µl α-labeled ^{32}P dATP @6000 Ci/mmol

17 µl ddH_20

1 µl terminal deoxytransferase (TdT), 13 U/µl (Stratagene®)

50 µl total volume

This mixture was incubated at 37°C for 1 hour. Each probe was purified from the unincorporated nucleotides by exclusion chromotograhpy using Sephadex™ G-25 matrix (see Spun-column chromatography for probe or large-fragment cleanup).

Random-primed probes were utilized only when a DNA template of sufficient size (100 nucleotides or larger) was available such that a set of random hexamers or decamers could be successfully used as primers to synthesize complementary strands while incorporating α-labeled ^{32}P-dCTP. A modification of this method used specific primers made complementary to the flanking regions of the interested area, thereby ensuring probes of a uniform size and a specific nucleotide sequence and usually with a substantially higher specific activity than end-labeled equivalents. Preparation for making these probes began with the heat denaturation of the double-stranded DNA so that the primers could be bound to the single-stranded DNA templates. First the double-stranded DNA template was mixed with all solutions except the enzyme and radioactive nucleotide (Promega's Prime-A-Gene™ Labeling System).

2 µl DNA (linear plasmid at 25 ng/µl)

2 µl dNTP mix (500 µM each: dATP, dTTP, dGTP)

15 µl primer buffer mix:

71 µl buffer: 1.59 g HEPES

1.7 ml 1 M Tris-HCl

0.170 ml 1 M $MgCl_2$

0.021 ml 2-mercaptoethanol

7.1 ml Total volume

16 µl primer (1 ug)

13 µl bovine serum albumin (BSA) at 10 mg/ml

100 µl Total volume of primer buffer mix

25 µl ddH_20

44 µl Preliminary total volume for probe mixture

This mixture was then denatured by heating it to 95°C for 3 minutes, followed by chilling on ice for 5 minutes. Five microliters of α-labeled ^{32}P-dCTP, 3000 Ci/mmol, and 1 µl Klenow fragment were then added. This complete mixture was incubated at room temperature (22-25°C) for 1 hour. After this time the probe was passed through a Sephadex™ G-50 column (see Size exclusion chromatography) to remove unincorporated nucleotides.

Quantification of each probe's specific activity was performed by the use of a Beckman™ LS7000 Liquid Scintillation System. Routinely, a 1µl aliquot of the probe was placed into a dry 0.6 ml centrifuge tube and analyzed by the scintillation counter to obtain an estimate of the disintegrations per minute/microliter (dpm/µl) probe. The dpm/µl was considered to roughly equal the counts per minute/microliter (cpm/µl) available from each prepared probe.

Spun-column chromatography for probe or large fragment cleanup

Spun-column chromatography (Sambrook *et al*., 1989) is the retarded migration of smaller molecules such as free nucleotides, very short oligonucleotides, and salts through a matrix while larger molecules pass quickly through during centrifugation. The larger molecules are excluded from holes in the matrix beads while the migration of smaller molecules is greatly inhibited. The DNA exclusion limit of Sephadex™ G-25 (Pharmacia Biotech) is 10 bp, whereas the exclusion limit of G-50 is 20 bp. G-25 was primarily used for purifying the end-labeled 25-mer oligonucleotides. Sephacryl® S-400 (Pharmacia Biotech) has an exclusion limit of approximately 271 bp and was used to selectively remove primer dimers (very short incomplete products) from PCR products.

The Sephadex™ G-25 or G-50 was equilibrated in TE buffer (5 g/ml) at 65°C for 1-2 hours. Fibers of sterile siliconized glass wool were used to plug the bottom (0.1 ml) of a 1 ml syringe. The syringe was then filled with the equilibrated Sephadex™ and placed into a 15 ml conical tube (the syringe should hang freely). The Sephadex™ was packed by centrifugation at 1,600 x g (2,700 rpm in a Sorvall™ T6000B swinging bucket rotor) for 4 minutes. Sephacryl® S-400 (pre-equilibrated with 20% ethanol) was packed by centrifugation at only 800 x g in order to avoid crushing the beads. If the volume of the packed resin was not between 0.9 to 1.0 ml, more Sephadex™, or Sephacryl®, slurry was added and the column centrifuged as before. Once the column was prepared, 100 µl TE buffer was added to the column and this was centrifuged as before. This wash was repeated again to ensure that the DNA sample would be eluted in TE buffer and not some other solvent (e.g. Sephadex™ storage buffer, PCR buffer or kinase buffer). The buffer from the bottom of the conical tube was removed and a sterile 1.5 ml microcentrifuge tube (de-capped) added to capture the probe. Sterile ddH_20 or TE buffer was added to

bring the probe or DNA sample (PCR product) up to 50 µl after incubation for the appropriate time. The 50 µl sample was then added to the top of the column and centrifuged as before. Since the purified DNA sample was collected into a de-capped microcentrifuge tube, it was transferred to a new tube so that it could be stored at –20°C. Quantification of the probe's specific activity was performed by use of a liquid scintillation counter. Quantification of a PCR product was done by submarine agarose gel electrophoresis.

Southern (1975) transfer of nucleic acids from agarose gels

Agarose gel electrophoresis of DNA molecules was used as a tool in order to visualize the apparent sizes, relative to their electrophoretic mobility, of restriction endonuclease-cut DNA fragments and/or uncut plasmid preparations. Further analyses of the DNA samples were made possible by transferring the DNA to membranes which were subsequently screened, sometimes repeatedly, by hybridization with isotopically-labeled probes. The common technique of DNA transfer from agar to a membrane, originally described by Southern (1975) and commonly referred to as the Southern transfer or blot, was modified and routinely used to preserve electrophoretic profiles of DNA for future analyses via hybridization (Sambrook *et al*., 1989).

DNA samples were gel electrophoresed, stained with EtBr and visually checked to ensure that the DNA profile appeared as expected. The gel was placed in an alkaline denaturing solution, 0.4 M NaOH, and gently rocked for 30 minutes. During this time, thick chromatography paper (Fisher Scientific), commonly called filter paper, and ordinary single-fold paper towels were cut to the size of the gel, usually 12 cm x 22 cm.

Transfer solution, 0.5 M NaOH, 0.5 M NaCl, was prepared and poured into a shallow tray to a depth of 2-3 mm. A very thin sponge, 14 cm x 26 cm x 0.5 cm, was then wetted and positioned in the middle of the tray. A large volume pipet (25 ml) was used as a "rolling pin" to gently squeeze out any air bubbles from underneath, or within, the sponge. Three pieces of pre-wetted filter paper were placed on top of the sponge, one piece at a time, while using the pipet to gently remove any trapped air bubbles. A nylon membrane, Biodyne® A (GIBCO BRL®), was cut to the exact size of the gel, labeled appropriately and submerged in the transfer solution.

The base of the Southern (1975) transfer setup was then ready for the agarose gel. The gel was placed upside down onto a 0.5 cm acrylic sheet and guided to the top of the blotting paper with care to not tear the gel. All air bubbles were removed from under the gel by gentle use of the pipet and gloved fingers. The pre-soaked membrane was placed on the top of the gel with the labeled-side facing it, and again any air bubbles removed as before. One sheet of wetted blotting paper was added to the stack, bubbles removed, and then a 4-6 cm high stack of cut paper towels placed on top. A light-weight object, such as a 12 cm x 22 cm x 1 cm piece of acrylic, was placed on top of the paper towels to apply even pressure. After 5 minutes the paper towels were removed and the wet ones were discarded. The remaining dry paper towels were again placed on top. The wicking action of the paper towels pulled the transfer solution through the sponge, blotting paper, gel, nylon membrane, blotting paper, and finally to the dry paper towels, carrying the DNA from the gel to the membrane.

Each hour the wet paper towels were removed and replaced with dry ones. After 3 hours the transfer was considered complete. The membrane was removed and soaked

in a neutralizing solution, 0.2 M Tris-HCl, pH 7.5, 2X SSC (20X SSC: 175.3 g NaCl and 88.2 g sodium citrate in 1 liter ddH_2O), for 15 minutes with gentle shaking. It was then dried on a pieces of filter paper, by moving it periodically to new sheets, at room temperature (72°F). It was usually dried overnight at room temperature, but occasionally it was baked at 85°C for 45 minutes. The dried membrane was subjected to short wave (254 nm) UV irradiation, at a distance of 10 cm, for 3 minutes to permanently affix the DNA.

Hybridization of radio-labeled probes to membranes

Membranes consisting of Biodyne® A or Gene Screen Plus™ were often utilized for the purpose of binding DNA, and were subsequently hybridized to determine if the region of interest was present. Binding of the probe occurred in a hybridization assay which consisted of the membrane, probe and Hybridization Solution incubated in a rotating hybridization tube. The Hybridization Solution (1X) consisted of 80.0 ml 50% PEG, 30.0 ml 20X SSPE (3.0 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA), and 276.0 ml 10% SDS. The hybridization tubes were large cylinders, Techne™ part number FHB11 (7 cm diameter by 22 cm long), which were designed to be slowly rotated within a temperature controlled oven, the Techne™ Hybridiser HB-1D®. The typical hybridization began with the wetting of a nylon membrane with 2X SSC (20X SSC: 175.3 g NaCl and 88.2 g sodium citrate in 1 liter ddH_2O). The membrane was then placed into a hybridization tube containing 10 ml Hybridization Solution. The membrane and Hybridization Solution were allowed to incubate at a moderately stringent hybridization temperature, usually 55 to 65°C, for 30 minutes. To add the appropriate

amount of probe, at least 1 million counts per minute (cpm) were mixed with ddH_2O to bring the volume up to 50 µl. If the probe was not an end-labeled oligonucleotide, and therefore double stranded, the 50 µl probe solution was then heat denatured at 100°C for 3 minutes before adding it directly to the hybridization tube. If the probe was an end-labeled oligonucleotide, it was heated to 100°C for one minute before adding directly to the Hybridization Solution.

Each hybridization was allowed to proceed for 16 to 20 hours. After the appropriate incubation period was complete, the excess Hybridization Solution and probe were poured into a radioactive waste bottle. The membrane was then transferred to a plastic tray containing about 200 ml of wash solution and allowed to rock gently back and forth. The typical wash solution consisted of 2.5X SSC, 0.1% SDS. Two washes were performed at room temperature, after which the membrane was placed on a sheet of filter paper and scanned with a hand-held survey (Geiger) meter. If the edges of the membrane were below 100-200 cpm, the radioactive "background" or non-specific binding of the probe was considered to be low enough to wrap the membrane in plastic wrap and expose it to film for autoradiography. If the background was considered to be too high, the membrane was washed in pre-warmed wash solution at the hybridization temperature for 10 minutes. If the background was still too high, the wash at hybridization temperature was extended to 30 minutes, or the temperature was raised by three degrees. If the film background was over-exposed, and the membrane had been wrapped well to prevent it from completely drying out, it was washed again at an even higher stringency. When a membrane needed to be hybridized with a different probe, or re-hybridized at a later date, the bound probe was removed by washing at 80°C for 20

minutes in strip solution (0.1X SSPE, 0.5% SDS). This technique would usually remove 90-95% of all bound probe.

Creation of a large insert Roseate Spoonbill genomic library in the Lambda DASH® II replacement vector

The creation of a Spoonbill genomic library in the Lambda DASH® II replacement vector was carried out by following the general protocol outlined in Glover and Hames, 1995, and the Stratagene® Instruction Manuals, 1997a & b. The Lambda DASH® II DNA pre-digested with *Bam*HI was supplied by Stratagene® as part of their Lambda DASH® II/*Bam*HI Vector Kit.

Spoonbill genomic DNA was isolated as described above from whole blood. A *Sau*3AI partial digestion procedure (pilot procedure) was then performed on a genomic DNA sample (30 µg) in order to estimate the amount of restriction enyzme needed to create fractions of DNA in the size range of about 9 to 23 kbp (the practical insertion limits into Lambda DASH® II/*Bam*HI arms). Sixty microliters DNA (0.5 µg/ul) was added to 840 µl TE, pH 8.0 and 100 µl 10X *Sau*3AI buffer. Ten 0.6 ml microcentrifuge tubes were lined up in a thick slurry of ice water to receive the diluted DNA and enzyme mixtures. Sixty microliters of the DNA solution was added to tube #1 and thirty microliters of DNA solution was added to the additional tubes numbered 2-10. One microliter (4 U/µl) of *Sau*3AI was added to tube #1, which provided 2 units of enzyme per 1 µg of DNA in tube #1 during the subsequent digestion period (see the following steps). The sample was mixed gently with a pipet tip and then a 30 µl aliquot was transferred to tube #2. After the sample was mixed gently, a 30 µl aliquot from this tube

was transferred to tube #3. After the sample was mixed gently, a 30 µl aliquot was transferred to tube #4 and the serial dilution was continued through tube #9. The 30 µl aliquot removed from tube #9 was discarded so that tube #10 remained a 30 µl uncut control (no enzyme added). All 10 tubes were placed in a 37°C water bath for the restriction endonuclease digestion to occur. After 1 hour, the enzyme was heat denatured by placing the tubes at 70°C for 15 minutes.

Seven microliters of 5X agarose gel electrophoresis loading buffer (25% glycerol, 0.5% sodium dodecyl sulfate, 0.1% bromophenol blue, 0.1% xylene cyanol, 50 mM EDTA) was added to each 30 µl sample. A thin 1% agarose base was prepared in an agarose gel tray. After the agarose solidified, a thicker 0.3% agarose solution was layered on top and allowed to solidify at 4°C. The ten 37 µl samples were then loaded into wells. A DNA sizing ladder (lambda DNA cut with *Hin*dIII) was added and the gel electrophoresed in 1X TBE buffer at 30 V and 4°C for 14 hours. Visualization of the DNA was accomplished by submerging the gel in a solution of EtBr (0.5 µg/ml) for 10 minutes followed by a 10 minute wash in deionized water and placement on a UV transilluminator.

Gel analysis of the cut DNA samples revealed that the *Sau*3AI serial dilution experiment resulted in the desired partial digestions of the Spoonbill DNA. Based upon these results, a preparative digestion of Spoonbill DNA was immediately begun. The preparative digestion cut 10 µg of DNA in each serially-diluted tube. One hundred eighty microliters of Spoonbill genomic DNA (0.5 µg/µl) was added to 2.250 ml TE, pH 8.0 and 270 µl 10X *Sau*3AI buffer for a total of 2.7 ml. Six hundred microliters was added to microcentrifuge tube #1 and 300 µl was added to 7 additional microcentrifuge

tubes, each in a thick slurry of ice water. Ten microliters (40 U) of *Sau*3AI restriction endonuclease was added to tube #1 and gently mixed to give a ratio of 20 units of enzyme per 10 µg DNA during the digestion (see the following steps). Three hundred microliters was transferred from tube #1 to tube #2, which was then mixed gently. The serial dilution was continued, each time transferring 300 µl to the ascending tube number until tube #8 was completed. The 300 µl taken from tube #8 was then discarded, leaving tube #9 as a negative control. All 9 tubes were placed at 37°C for 1 hour. The digestion was terminated by transfer to 70°C for 15 minutes, inactivating the enzyme. Fifteen microliters (0.5 µg) of DNA was removed from each tube and mixed with 4 µl 5X agarose gel loading buffer. The total volume (19 µl) of each aliquot was loaded onto a 0.5% agarose gel supported by a 1% agarose base. Submarine gel electrophoresis was performed at 30 V and 4°C for 15 hours. Visualization of the DNA was accomplished by submerging the gel in a solution of EtBr (0.5 µg/ml) for 10 minutes followed by a 10 minute wash in deionized water.

The remaining volume from each sample (285 µl) was mixed well with an equal volume (285 µl) of phenol:chloroform and centrifuged for 2 minutes at 10,000 x g. The phenol:chloroform was removed and the extraction repeated with fresh phenol:chloroform. Each supernatant was then transferred to a new tube. Eight hundred microliters of -20°C 100% ethanol and 30 µl 3 M sodium acetate were added to each tube. The tubes were mixed well and centrifuged at 10,000 x g at 4°C for 10 minutes. Each supernatant was removed with a different drawn-out Pasteur™ pipet with extreme care to not disturb any pelleted DNA. One milliliter of -20°C 70% ethanol was gently added to each tube, each tube was gently inverted once, and the tubes were centrifuged at

10,000 x g for 5 minutes at 4°C. Each supernatant was removed with a drawn-out Pasteur™ pipet and the DNA pellet was dried in a Savant Speed Vac™ vacuum concentrator for 2 minutes. Fifty microliters of TE, pH 8.0 was added to each tube and vortexed gently to resuspend the DNA.

A continuous sucrose gradient, 10 to 40%, was prepared as follows. Sucrose was added to a buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA) to create the 10, 15, 20, 25, 30, 35, and 40% solutions. Five milliliters of the 40% solution was added to the bottom of two 35 ml capacity polyallomer ultracentrifuge tubes. Five milliliters of each sequentially diluted sucrose solution was gently added to the top of the more concentrated solution. This created two step gradients, which were then placed at 4°C for 12 hours in order to allow the sucrose interfaces to diffuse and create a continuous gradient. The top 3 ml of each gradient was removed in order for the DNA sample or balance solution to be added. The 50 µl samples containing DNA in the size range of 3,000 to 23,000 bp were combined (250 µl) and placed on top of one of the sucrose gradients. The second gradient was used as a balance by adding 10% sucrose to the top. The tubes were placed in a Sorvall™ AH 629 swinging bucket rotor and centrifuged in a Beckman™ L8-70 Ultracentrifuge. The recommended centrifugation time (Glover and Hames, 1995) was 26,000 rpm for 24 hours for a total of 37,440,000 revolutions.

The Beckman Recovery System® was used to puncture the bottom of the DNA-containing sucrose gradient tubes and collect the contents. Fractions of approximately 1 ml were collected by gravity into 33 tubes. Eighteen microliters from each sample was mixed with 3.5 µl 6X sucrose loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose) and loaded onto a 0.5% gel (poured on a very thin 1% base). The

DNA was electrophoresed at 30 V for 14 hours and stained by EtBr. After a 1 hour destain of the gel, and a 75 second exposure to Polaroid type 55 film, the faint smearing pattern of DNA could be discerned on the film negative. Fractions in the size range of 9 to 23 kbp were chosen to be recovered and purified.

The sucrose was removed from the samples by affinity column chromotography. A NENSORB™ 20 (Dupont™) nucleic acid purification cartridge was wetted by the addition of 2 ml 100% methanol, followed by a 2 ml rinse with Reagent A (100 mM Tris-HCl, 10 mM triethylamine, 1 mM disodium EDTA, pH 7.7). Each of the 1 ml sucrose samples (10-14) was supplemented with 1.4 µl Reagent A, then loaded onto the column. Three milliliters of Reagent A was added to wash the column, followed by a 4 ml wash with water. The elution of the DNA began with the addition of 1 ml Reagent B (50% methanol) to the top of the column. Three fractions, of approximately 300 µl each, were collected into 1.5 ml microcentrifuge tubes. The DNA containing microcentrifuge tubes were placed in a Savant Speed Vac™ vacuum concentrator for 4 hours or until the samples had dried completely.

The dried DNA samples were resuspended in TE, pH 8.0 and combined to yield a total volume of 100 µl. One-half of the entire sample (50 µl) was analyzed in a Beckman™ DU®-40 Spectrophotometer. Additionally, a 5 µl sample was loaded on a 0.5% (1% base) agarose gel and subjected to electrophoresis at 20 V for 12 hours. The gel was stained in EtBr and destained in water. A smear of DNA from about 7-23 kbp should be observed. After verification of the recovery of properly sized DNA, a 25 µl volume was diluted to 100 µl with ddH_2O and ethanol precipitated by the addition of 11 µl 3 M sodium acetate and 350 µl -20°C 100% ethanol. After centrifugation at 10,000 x

g for 10 minutes at 4°C, the ethanol was removed with a drawn-out Pasteur™ pipet and 500 µl of -20°C 70% ethanol was added. The tube was inverted once and then centrifuged at 10,000 x g for 5 minutes at 4°C. The ethanol was then removed with a drawn-out Pasteur™ pipet and the pellet was dried for 2 minutes in a Savant Speed Vac™ vacuum concentrator. The DNA was resuspended in 4 µl ddH_2O to be used in a ligation reaction with Lambda DASH® II vector pre-cut with *Bam*HI.

The ligation of size-selected DNA to the Lambda DASH® II/*Bam*HI vector was performed by combining the following ingredients.

1 µl	Lambda DASH® II predigested with *Bam*HI (1 µg)
2 µl	size-selected Spoonbill genomic DNA digested with *Sau*3AI (0.34 µg)
0.5 µl	10X ligase buffer (500 mM Tris-HCl, pH 7.5, 70 mM $MgCl_2$, 10 mM dithiothreitol (DTT))
0.5 µl	10 mM ATP (freshly made stock)
0.5 µl	ddH_2O
0.5 µl	T4 DNA ligase (GIBCO BRL®, 5 U/µl)
5 µl	Total volume

This ligation setup was incubated at 4°C for 17.5 hours. A 1 µl aliquot of the ligation was then added to a quickly thawed 25 µl aliquot of Gigapack® III Gold packaging extract (Stratagene®, 1997) and mixed gently. After the mixture was allowed to incubate at room temperature (21°C) for 2 hours, 500 µl of SM buffer (5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 50 ml 1 M Tris-HCl, pH 7.5, 5 ml 2% w/v gelatin in 1 liter ddH_2O, autoclaved) and 20 µl fresh chloroform were added so it may be stored at 4°C. The

packaging procedure was repeated to ensure a sufficient amount of viable lambda clones had been created.

The following procedure describes how the packaged library was titered and subsequently amplified to make a large quantity stock for future screening procedures. An MRA(P2) colony was transferred with a sterile inoculation loop to 5 ml NZY broth and grown to an absorbance reading (OD_{600}) of 0.5. The bacterial culture was then centrifuged at 500 x g for 10 minutes and resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5. One microliter of the packaged library and 1 microliter of a 1:10 dilution of the library were mixed with 200 µl of freshly resuspended MRA(P2) cells in separate sterile 10 ml culture tubes. The phage were allowed to attach to the bacteria during a 15 minute incubation period at 37°C. A 100 ml of NZY top agarose was melted in the microwave and sterile water replaced that which boiled off as steam. It was then set in a 48°C water bath and time was allowed for the temperature to equilibrate. A 3 ml aliquot of the top agarose was added to each tube. The tube was then immediately mixed and its contents gently poured evenly onto an 82 mm NZY plate. The plates were allowed to solidify at room temperature for 30 minutes before being placed upside down in a 37°C incubator for 12-16 hours.

The number of plaques visible in the bacterial lawn was used to calculate the number of plaque forming units present in the test sample. Each plaque represented a plaque forming unit and this was multiplied by the dilution factor. The 1 µl sample of 10-fold diluted phage gave 400 plaques. This number corresponds to approximately 4,000 plaque forming units per microliter of packaged phage. NZY agar plates were prepared in 150 mm Petri dishes and partially dried by incubating the plates at 37°C for

12-16 hours. Confluently lysed plates represented the most efficient method for screening large quantities of phage without creating too many plates. By adding 12.5 µl of packaged phage to 600 µl of washed and resuspended MRA(P2) cells, the mixture could be incubated at 37°C for 15 minutes, mixed with 6.5 ml top agarose (warmed to 48°C), and plated out on 150 mm Petri dishes. The confluently lysed plates (15) were then overlayed with 10 ml SM buffer and incubated at 4°C for 16 hours. The SM/phage solution was removed from each plate, and these were each washed with an additional 2 ml SM buffer. All solutions (170 ml total volume) were collected in a clean (250 ml) GSA centrifuge bottle. Four milliliters of chloroform was added to the SM/phage solution and allowed to sit at room temperature for 15 minutes. The sample was centrifuged at 500 x g for 5 minutes, after which the supernatant (approximately 170 mls) was transferred to a new 250 ml GSA bottle.

The titer of the lambda phage solution was determined by sequential ten-fold dilutions. Freshly prepared MRA(P2) cells were mixed with different phage dilutions and spread with NZY top agarose on 82 mm Petri dishes. The dishes were incubated at 37°C for 12-16 hours, followed by an enumeration of the plaques. The dishes representing the 10,000,000 (10^{-7})-fold dilution yielded 450 plaques. This was calculated to equal a titer of 4.5×10^9 pfu/ml. Aliquots of the lambda library were stored in 15 ml polypropylene tubes with 7% v/v dimethylsulfoxide (DMSO) at –80°C.

Large scale preparation of a lambda clone

Once a desired lambda clone (isolated plaque) was identified from a screened library, a large scale culture of the phage was produced in order to obtain sufficient

quantities of DNA for sequence determination and other characterizations, such as restriction mapping. NZY medium was prepared by mixing together the following components in a total volume of 1 liter deionized water, adjusting the pH to 7.5, followed by autoclaving for 35 minutes.

10.0 g NZ amine (casein hydrolysate-enzymatic)

5.0 g NaCl

5.0 g yeast extract

2.0 g $MgSO_4 \cdot 7H_2O$

A selected lambda clone (plaque) was estimated to contain approximately 10^6 plaque forming units when eluted into 1 ml. This is far less than the 10^{10}-10^{11} pfu/ml needed to successfully prepare a large quantity of lambda phage. Therefore, the following amplification procedure was performed. The plaque was stored in 1 ml SM buffer (5.8 g NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, 50 ml 1 M Tris-HCl, pH 7.5, 5.0 ml 2%w/v gelatin in a final volume of 1 liter deionized H_2O and autoclaved for sterility) with 20 µl fresh chloroform. Confluently lysed plates (2) were created by first incubating 100 µl phage with 200 µl of MRA-prepared cells (grown in NZY broth to absorbance 0.5 at 550 nm then washed and resuspended in 10 mM $MgSO_4$) at 37°C for 20 minutes. Then the phage/bacteria mixture was added to 3.0 ml NZY top agarose (melted and heated to 48°C) and poured onto dried and warmed NZY plates. The plates were allowed to incubate at 37°C for 12-16 hours. If the bacterial lawn was completely lysed by the lambda phage then 5 ml SM buffer was added to each plate and allowed to incubate at room temperature with gentle rocking for 2 hours. The SM buffer/phage suspension was then transferred from the plates to a sterile 15 ml polypropylene tube and 100 µl

chloroform was added. This phage suspension was stable at 4°C for at least a month. Dimethylsulfoxide (7% final concentration) was added to some of the phage suspension and this was stored at –70°C. A useful phage titer is typically 10^{10}-10^{11} pfu/ml.

The highly titered (10^{10}-10^{11} pfu/ml) lambda clone was then used to create a large quantity of that particular clone. This was accomplished by performing a 500 ml bulk preparation of the lambda phage clone. One milliliter of an overnight growth of MRA cells was added to 500 ml NZY broth supplemented with 0.2% $MgSO_4$. The MRA was allowed to grow to an OD of 0.3. Approximately 2.6 x 10^{10} pfu were added to the growing MRA cells. This mixture was allowed to incubate at 37°C and shake at 250 rpm for approximately 16 hours. If considerable amounts of cell debris were easily seen (a "splintery" appearance instead of a typical culture), then the procedure was continued by the addition of 10 ml chloroform followed by shaking for 10 minutes.

The flask was then removed and allowed to cool to room temperature (approximately 10 minutes). DNase and RNase were added to obtain a final concentration of 1 microgram/ml of each. This mixture was incubated at room temperature for 30 minutes followed by the addition of 29.2 g NaCl, which was hand agitated to dissolve completely. The mixture was then divided equally into two 500 ml (Sorvall™ GS-3) centrifuge tubes and allowed to incubate in an ice/water bath for one hour. Centrifugation was then performed at 11,000 x g (8,070 rpm) in a GS-3 rotor for 10 minutes at 4°C. The supernatants were combined into the same 1000 ml flask in order to remove the majority of the chloroform before adding the PEG, which will extract into the chloroform. PEG (molecular weight of 8,000) was added to yield a 10% final concentration (55 grams) and dissolved by slow stirring with a stir bar on a magnetized

stir plate at room temperature. After the PEG totally dissolved (1 hour) the suspension was equally divided into GS-3 tubes, which were then placed in an ice/water bath for a minimum 2 hours (overnight, when convenient). Centrifugation was performed at 11,000 x g (8,070 rpm) in a GS-3 rotor for 10 minutes at 4°C. The supernatant was then poured off and the tubes allowed to completely drain of excess fluid for 5 minutes.

The PEG/phage pellets were resuspended in 6.5 ml SM buffer by gently rocking (not vortexed) the tubes at room temperature until the pellets were totally resuspended (approximately 20 minutes). The SM buffer/phage solution was then transferred to a 50 ml polypropylene tube and a 4.5 ml volume of chloroform (fresh) was added to each tube, which was then gently shaken for 1 minute. The tubes were centrifuged at 3000 x g (4,220 rpm) for 15 minutes at 4°C. The supernatant was moved to a glass graduated cylinder so that the phage suspension could be inspected. A cloudy suspension would usually reflect the high titer of phage, whereas an almost clear suspension would indicate a low phage titer, which probably would not be sufficient. If the titer appeared fine, then 0.75 g cesium chloride per ml of phage suspension was added and dissolved by inverting the mixture repeatedly. The samples were then loaded into ultracentrifuge tubes so that they may be centrifuged at 38,000 rpm at 20°C for 24 hours.

Identification and collection of the lambda phage is dependant upon the concentration and optical nature of the phage. If phage are present in high enough quantity, and the equilibrium density gradient of the cesium chloride formed properly, then concentrated phage should appear as a light blue band across the middle region of the ultracentrifuge tube. Each ultracentrifuge tube (2) was set up in an elevated clamp with a liquid disposal container beneath to catch the dripping cesium chloride. Two 21-

gauge needles and one syringe were used to gently remove the blue band from each tube. One needle was inserted through the top of the tube to act as a vent so the phage can be removed by the other needle/syringe combination. The second needle was attached to the syringe, which was checked for free motion of the plunger before use, and then inserted into the side of the tube approximately 0.5 cm beneath the phage band. Slowly and carefully the phage band was pulled into the syringe. The phage sample was then ready to be dialyzed to remove the cesium chloride. Two 8 cm lengths of dialysis tubing (diameter of 15 mm) were sealed with dialysis clips or silk thread at one end. The phage/cesium chloride samples were transferred to the tubing, which were then sealed by clipping the other ends. Care was taken to remove all air bubbles and leave extra space for the samples to expand as a result of osmosis. The dialysis samples were submerged in 1 liter of dialysis buffer (1.17 g NaCl, 4.06 g $MgCl_2$, 100 ml 1M Tris-HCl, pH 8.0, in 2 liters) for 4 to 12 hours with stirring at 4°C and then transferred to fresh dialysis buffer for another 4 hour treatment. The dialyzed phage suspensions were transferred to 1.5 ml microcentrifuge tubes in 400 µl volumes. EDTA (ethylenediamine tetraacetic acid, disodium form) was added to each tube to create a final concentration of 20 mM (pre-made 0.5 M EDTA, pH 8.0 was used). Proteinase K was added to each tube to a final concentration of 100 ug/ml. SDS (sodium dodecyl sulfate) was added to a final concentration of 0.5% (from a 10% solution). This mixture was inverted multiple times in order to mix it well and then incubated 56°C for 1 hour.

Organic extraction of proteins was then performed by the addition of an equal volume distilled & equilibrated (with TE buffer, pH 8.0) phenol to each microcentrifuge tube. Each tube was inverted several times to create an emulsion, and then centrifuged at

10,000 x g for 3 minutes. A 100 µl pipettor was used to remove most of the phenol (lower phase), which was discarded into an organic waste container. An equal volume phenol:chloroform (50:50) mixture was added to each microcentrifuge tube. Each tube was inverted several times to create an emulsion, and centrifuged at 10,000 x g for 3 minutes. The aqueous phases (upper layer of each) were then transferred by use of a mechanical pipettor to sterile microcentrifuge tubes, with care to leave behind the phenol:chloroform interphase. Five hundred microliters diethyl ether was added to each tube and mixed by inverting repeatedly. The tubes were then centrifuged at 10,000 x g for 15 seconds. The ether was removed with a drawn-out Pasteur™ pipet and the tubes were left open in a vented hood for 5 minutes to allow residual ether to evaporate.

Ethanol precipitation of the DNA samples began by the addition of 0.1 volume 3.0 M sodium acetate to each microcentrifuge tube. Approximately 2.5 volumes of -20°C 100% ethanol was added to each tube, which were then place in a -50°C ethanol bath for 2 minutes. The tubes were centrifuged at 10,000 x g for 10 minutes. The supernatant was removed with drawn-out Pasteur™ pipets. Five hundred microliters -20°C 70% ethanol was added to each tube and the tube inverted once, making sure to not disturb the DNA pellet. The tubes were centrifuged at 10,000 x g for 5 minutes, after which the supernatant was removed with a drawn-out Pasteur™ pipet. The samples were placed in a Savant Speed Vac™ vacuum concentrator for 5 minutes to completely dry the DNA. One hundred microliters of TE, pH 8.0 was added to each sample and DNA quantity and quality evaluations were performed on each.

Colony/Plaque Screen™ membranes for library screening and replica plating

Colony/Plaque Screen™ Hybridization Transfer Membranes (NEN™ Life Science Products) were used to remove a representative amount of plaques from an agar plate by placing a dry membrane on a pre-chilled plate, 1 hour at 4°C, for 2 minutes. This first lift was called the 'A' lift in anticipation of performing a second, 'B', lift. During the incubation period, a 21-guage needle was dipped into India ink and used to place a series of asymmetric holes around the perimeter of the membrane and plate. To denature the DNA, the membrane was placed with the DNA side facing up in a 0.75 ml pool of 0.5 N NaOH for 2 minutes. It was blotted of excess fluid by placing it on thick filter paper for 10 seconds. It was transferred to a fresh 0.75 ml pool of 0.5 N NaOH for 2 minutes, then again blotted on filter paper for 10 seconds. The alkaline membrane was neutralized by transferring it to a 0.75 ml pool of 1.0 M Tris-HCl, pH 7.5 for 2 minutes, followed by blotting for 10 seconds. This was repeated once more with 1.0 M Tris-HCl and blotted dry. The membrane was allowed to completely dry at room temperature for 2 hours, or at 80°C for 30 minutes, to affix the DNA before being subjected to hybridization with radioactive probes.

To create the second lift (B), a new membrane was placed on the same plate and marked with the needle in the same places as the 'A' lift. This membrane was then treated by the same method as the 'A' lift to denature and affix the DNA. All membranes were pre-wetted with 2X SSC before insertion into a hybridization tube with buffer.

Replica plating was also performed to be able to screen all of the colonies of a transformation and still be able to grow the original cells, via rapid plasmid prepartion, for DNA sequence determination of the cloned DNA. The first membrane, 'A', was

placed on a chilled (1 hour at 4°C) plate with colonies for 2 minutes and the orientation marked with India ink. The lift was then transferred to a new agar plate containing antibiotic, with the colony side facing up. A second membrane, 'B', was pre-wetted by placement on a fresh agar plate, then placed against the colonies on the master filter. Orientation marks were added, relative to the 'A' lift. The 'B' lift was then placed, bacterial side up, onto a fresh agar plate containing antibiotic. Both plates with filters were incubated overnight at 37°C. Once the colonies had grown sufficiently, one lift was treated, as above, with NaOH to denature the DNA followed by Tris-HCl and drying to permanently affix the DNA. The second plate and membrane were sealed with PARAFILM® and stored at 4°C until needed. The treated lift would be hybridized to determine which colonies, by autoradiograph, carried the desired DNA insert. The colony indicated by a dark spot (positive) on the autoradiograph would then be identified on the second plate/lift by aligning the India ink markings.

During the replica lift procedure some of the colonies would be lost, literally or by smearing too much and mixing with other colonies. Because of this problem the replica procedure was modified. Often, only 10 to 20 colonies were grown on each transformation plate. Instead of using two membranes per plate, all of the white colonies from multiple plates were transferred to duplicate master plates by use of sterilized toothpicks. A master plate grid (Fig. 2) was used as a template for each plate during the replication process (see diagram below). Both master plates were incubated overnight at 37°C. A single lift was performed on one of the master plates and immediately treated with NaOH, as directed above, neutralized with Tris-HCl and dried completely. Membranes were then screened with the appropriate probes.

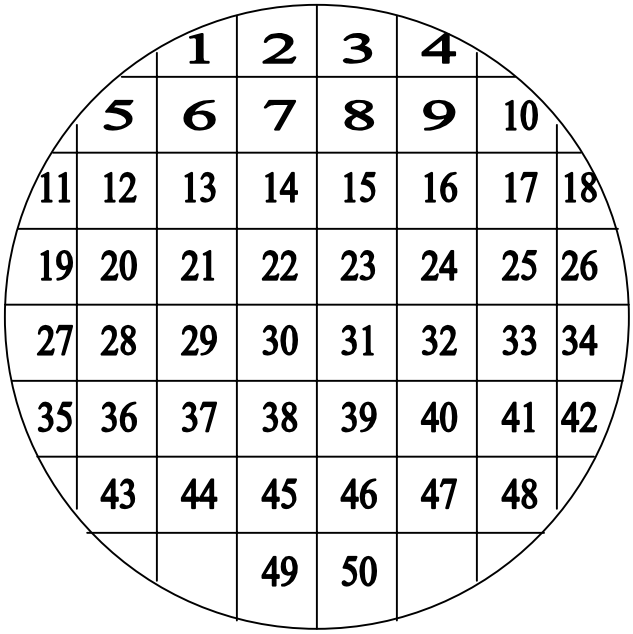

Figure 2. **Master plate grid.** Portions of bacterial colonies were transferred to specific numbered locations by use of a sterilized toothpick. Additional colonies were sometimes added to each plate by inserting them between the numbers to increase the total to approximately 90.

Bacterial screening by colony PCR

A quick survey of bacterial colonies on a plate is sometimes necessary and useful to ascertain the sizes of DNA inserts cloned into each plasmid's MCS. It is possible to select a colony containing the desired plasmid, harboring a specific insert size, from a mixture of colonies by creating a master plate of bacterial colonies at the same time as PCR amplifying the insert contained within the MCS. A 0.5 ml microcentrifuge tube containing 80 µl TE, pH 8.0 was prepared for every colony to be screened. An LB plate containing the appropriate antibiotic was prepared to act as a master plate for the selected colonies.

A sterile toothpick was used to touch an isolated colony. This was then used to gently scrape a specific region of the master plate, and finally to inoculate the corresponding 0.5 ml microcentrifuge tube containing TE buffer. After the required colonies were collected, the master plate was placed in a 37°C incubator for overnight growth. The inoculated TE buffer tubes were placed in a Perkin Elmer™ Cetus DNA Thermal Cycler for 10 minutes at 100°C and the resulting extracts (cell lysates) used for PCR amplifications in the following fashion. A new set of thin-wall (PCR) reaction tubes was set up with each containing the following:

2 µl DNA (heated cell lysate)

1 µl 10X *Taq* polymerase buffer (FisherBiotech™)

0.8 µl T3 primer (5 µM)

0.8 µl T7 primer (5 µM)

0.6 µl dNTP mix (2.5 mM each)

0.05 µl *Taq* polymerase (5 U/µl) (FisherBiotech™)

4.75 µl ddH_2O

10 µl Total volume

The parameters for the thermal cycle reaction were: 94°C for 2 minutes, followed by 29 cycles of 94°C for 50 seconds, 53 °C for 50 seconds, and 72°C for 50 seconds, followed by a cleanup temperature hold of 7 minutes at 72° and ending with a hold of 4°C. The resulting PCR amplification products were analyzed by gel electrophoresis to determine insert size, relative to the expected size of T3/T7 PCR amplification (approximately 220 bp) without an insert.

STR isolation from Spoonbill genomic DNA via enrichment technique utilizing biotin-labeled oligonucleotides and streptavidin-coated paramagnetic particles

Many STR-encoding DNA segments were isolated from the Spoonbill genome by selecting (enrichment) for them with the use of Streptavidin MagneSphere® Paramagnetic Particles (Promega). This enrichment procedure was adapted from papers by Li *et al.* (1997) and Gardner *et al*. (1999). Since the frequency of STR loci in avian species has been suggested to be as much as 10 times less than that observed in mammals (Weber and May, 1989; Crooijmans *et al*., 1993), the following protocol was used as a guide to isolate as many STR loci as possible from the Spoonbill genome.

Genomic DNA from 5 different Spoonbills (1 µg from each) was digested with *Sau*3AI (8 U) for 2 hours at 37°C in a total volume of 30 µl. The digested DNA was extracted with phenol, then ethanol precipitated and resuspended in 10 µl ddH_20. The total volume of DNA was gel electrophoresed in 1% low melting point (LMP) agarose (BRL®) with 1X TAE buffer (1X: 0.04 M Tris-acetate, 0.001 M EDTA) at 40V for 1.5

hours. A size standard consisting of pBR322 plasmid, digested with *Hin*fI, was run on the same gel. The fragments in the range of 300-800 bp were removed from the gel by use of a new razor blade and placed into a tared microcentrifuge tube.

The DNA in the gel slice was then purified from the agarose by use of GELase™ Agarose Gel-Digesting Preparation (Epicenter®) and the supplied 50X reaction buffer. Incubation was allowed to proceed at 45°C for 2 hours before cleanup commenced thereafter. The cleanup was accomplished by adding equal amounts of TE-equilibrated phenol and extracting twice. Diethyl ether was then used to extract the remaining phenol from the samples, followed by a standard ethanol precipitation (0.1 volume of 3.0 M sodium acetate with 2.5 volumes -20°C, 100% ethanol) in a 4°C microcentrifuge and washed with 1 ml –20°C 70% ethanol. The DNA was resuspended in approximately 50 µl ddH_20, and 5 µl was gel electrophoresed to visualize a successful precipitation procedure. Since oligonucleotide adapters (short duplexes with one cohesive end and one blunt end) were needed, they were prepared as follows.

The S62/S61 hybrid adapter (Gardner *et al*., 1999) consisted of the following oligonucleotides, with S62 containing a *Sau*3AI overhang (underlined):

S61: 5'-GGCCAGAGACCCCAAGCTTCG-3'

S62: 5'-<u>GATC</u>CGAAGCTTGGGGTCTCTGGCC-3' (*Sau*3AI overhang)

The *Sau*3AI overhang allowed the S61/S62 adapter to complement the *Sau*3AI-digested genomic DNA (Fig. 3). In later attempts to optimize the adapters, they were modified to ligate to *Tsp*509I or *Hin*P1I restriction endonuclease sites (underlined):

S63: 5'-<u>AATT</u>CGAAGCTTGGGGTCTCTGGCC-3' (*Tsp*509I overhang)

S64: 5'-<u>CGCG</u>AAGCTTGGGGTCTCTGGCC-3' (*Hin*P1I overhang)

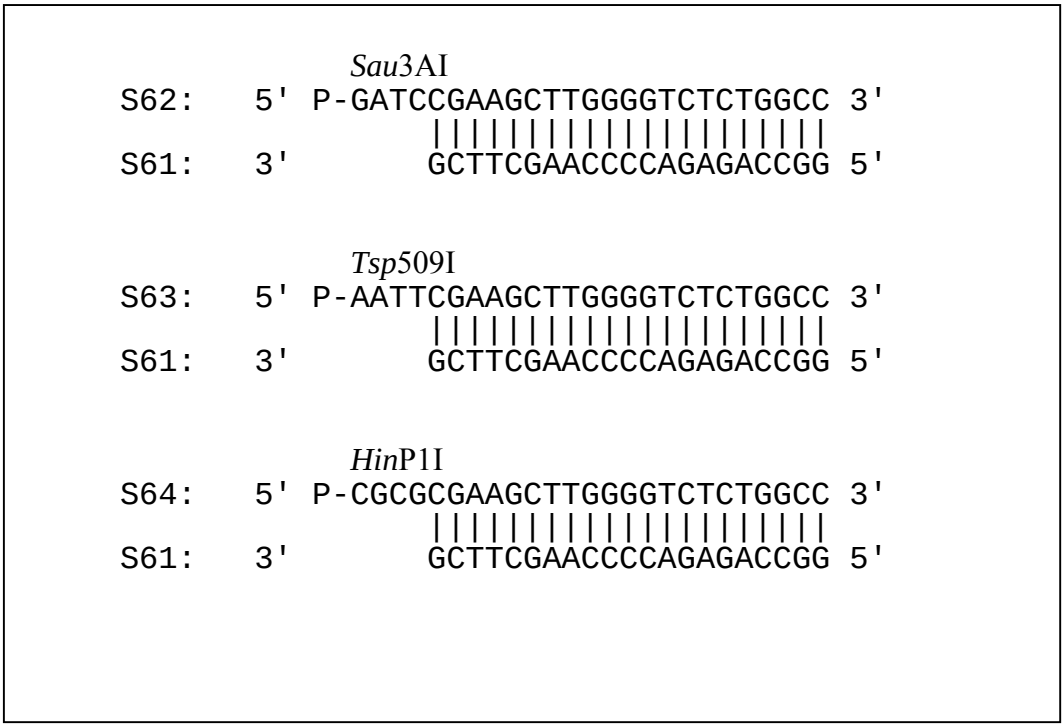

Figure 3. **Diagram of the oligonucleotide adapters.** Each adapter has one unique restriction endonuclease site. The oligonuceotides S62, S63 and S64 were phosporylated on the 5'-end to allow the ligation of each completed adapter to restriction endonuclease digested DNA. The "P" represents a molecule of adenosine-triphosphate (ATP) which was covalently linked to a specific oligonucleotide during a phosphosphorylation procedure.

Each new oligonucleotide was still complementary to S61 (Fig. 3). The S63/S61 hybrid produced an overhang (*Tsp*509I) which was compatible with an *Eco*RI-digested DNA. The S64/S61 hybrid produced an overhang (*Hin*P1I) which was compatible with a *Cla*I-digested DNA. The adapters were prepared for ligations to digested genomic DNA by the addition of a phosphate to the 5'-end of the oligonucleotides S62, S63, or S64 (each one separately).

Phosphorylation of oligonucleotides was set up as follows:

X µl	oligonucleotide (1.5 nmol)
1.25 µl	20 mM ATP
2.5 µl	polynucleotide kinase 10X buffer (New England Biolabs)
1.2 µl	polynucleotide kinase (12 U, New England Biolabs)
Y µl	ddH_2O
25 µl	Total volume

This mixture was incubated at 37°C for 1 hour, followed by kinase inactivation by heating at 65°C for 20 minutes. The adapter construction was completed by the addition of S61 (1.5 nmol) to each kinased oligonucleotide. This combination was then heated to 80°C for 2 minutes and allowed to slowly cool to room temperature over 1 hour.

The annealed adapter was ligated to restriction endonuclease-digested DNA. From this point, the procedure will reference the use of the S61/S62 adapter only.

Ligation Mixture:

45 µl size-fractionated *Sau*3AI-cut DNA

17 µl kinased S62/S61 hybrid adapter (750 pmol)

3 µl ddH_20

20 µl 5X ligation buffer: 250 mM Tris-HCl (pH 7.6)

50 mM $MgCl_2$

25% (w/v) polyethylene glycol-8000

5 µl ATP (20 mM)

5 µl DTT (20 mM)

5 µl T4 DNA ligase (GIBCO BRL®)

100 µl Total volume

This mixture was allowed to incubate overnight at 16°C. An equal volume of phenol was added and extracted twice, followed by an ether extraction and ethanol precipitation (see above). The DNA was resuspended in 38 µl ddH_20. Alternatively, the ligation reaction was passed through a Sephacryl™ S-400 column (see Spun-column chromatography for probe or large fragment cleanup) and approximately 40-45 µl recovered. The DNA solution collected from the column was desiccated *in vacuo* for 2 minutes to remove any residual ethanol.

The size-selected DNA fragments with ligated adapters, together known as constructs, were then PCR amplified in order to increase the concentration of the ones with adapters. The PCR mixture included:

5 µl DNA/adapter ligation

5 µl 10X Buffer A (FisherBiotech™)

8 µl S61 primer (5 µM)

3 µl dNTPs (2.5 mM each dNTP)

1 µl *Taq* polymerase (FisherBiotech™) 5 U/µl

28 µl ddH_20

50 µl Total volume

Filtered mineral oil (38 µl) was added to prevent liquid volatilization. The thermal cycle parameters were programmed into a Perkin-Elmer™ Cetus DNA Thermal Cycler:

94°C, 3 minutes

60°C, 45 seconds

72°C, 1 minutes

then linked to 29 cycles of:

94°C, 45 seconds

60°C, 45 seconds

72°C, 45 seconds

then linked to 72°C for 5 minutes, followed by 4°C soak. Routinely, a 1% agarose gel analysis of a 5 µl PCR sample would show a considerable smear on the gel in the range of the varying expected sizes.

The capture of STRs proceeded by the use of biotinylated oligonucleotides and Streptavidin MagneShere® Paramagnetic Particles (Promega). The beads were prepared for STR capture by beginning with a wash. One microcentrifuge tube (0.6 ml) of beads was gently resuspended flicking the tube repeatedly. The protocol by Promega recommends not to vortex the beads and to check for clumping, which would indicate the beads were no longer viable. One hundred microliters of the resuspended beads were transferred to a 0.6 ml microfuge tube. These beads were then washed three times, 300 µl per wash, with 0.5X SSC (20X SSC: 175.3 g NaCl and 88.2 g sodium citrate in 1 liter

ddH_2O) by using Promega's MagneSphere® Technology Magnetic Separation Stand. To make a 1.2 ml solution of 0.5X SSC, 30 µl 20X SSC was added to 1.17 ml ddH_20.

The beads were then resuspended in 100 µl 5X SSC, 200 pmol STRs, which was prepared as follows (capture with a single STR works well too):

50 pmol STR-1 (10 µl of a 5 µM solution)

50 pmol STR-2 (10 µl of a 5 µM solution)

50 pmol STR-3 (10 µl of a 5 µM solution)

50 pmol STR-4 (10 µl of a 5 µM solution)

35 µl ddH_20

25 µl 20X SSC

100 µl Total volume

Oligonucleotides labeled STR-1 through -8 have biotin at the 5'-end. Oligonucleotide STRs -9 through -12 are 3'-biotin-labeled. Additional oligonucleotides, PSTR-1 through -8, were synthesized identically as the "STR-" with the same number, except they were not biotin-labeled at the 5'-end (sequences not shown). The non-biotin-labeled PSTRs were created to be used as end-labeled probes. The biotin-labeled oligonucleotides (Table I) were generously donated by Bio-Synthesis Inc., Lewisville, TX.

The beads were hybridized (beads/STRs/SSC) at room temperature for 15 minutes with periodic gentle shaking. The solution containing unbound biotin-labeled oligonucleotides was washed away by 5X SSC, 100 µl per wash, 3 times. The beads were resuspended by gently flicking them in 50 µl 1X Hybridization Solution (0.5 M NaCl, 4% PEG), followed by an incubation at 55°C to pre-warm them for the addition of the DNA.

Table I. **Biotin-labeled STR oligonucleotides.**

STR-1	biotin-GAATAT$(GGAT)_4$	
STR-2	biotin-GAATAT$(GAYA)_4$	Y=T/C
STR-3	biotin-$(GAT)_9$	
STR-4	biotin-$(AAAG)_6$	
STR-5	biotin-$(AACT)_6$	
STR-6	biotin-$(GCA)_9$	
STR-7	biotin-$(CAC)_8$	
STR-8	biotin-$(CCT)_8$	
STR-9	$(CA)_{15}$-biotin	
STR-10	$(CT)_{15}$-biotin	
STR-11	$(AAT)_{12}$-biotin	
STR-12	$(AAC)_{12}$-biotin	

The constructs were then prepared for hybridization with the beads/STR oligonucleotides. Ten microliters (~100 ng) from the PCR amplification constructs was used for the capture technique. It was added to 38 µl Hybridization Solution and 2 µl oligonucleotide S61 (10 pmol/µl). This mixture was then heat denatured for 5 minutes at 95°C and immediately added to the beads suspended in 1X Hybridization Solution at 55°C. The beads/construct mixture was incubated at 55°C for 20 minutes to 1 hour with periodic gentle mixing. The hybridization time was increased to 3 or 4 hours during some isolations, with some success, to encourage more STR-containing fragments to hybridize. After the appropriate time of incubation, the beads were washed 4 times at

room temperature with 2X SSC, 10 pmol S61. Then they were washed 4 times at room temperature with 1X SSC, 10 pmol S61 to be sure the excess (unbound) DNA was removed. The washes were sometimes performed at the hybridization temperature (usually 55°C) in order to remove any of the weakly bound STR loci.

The putative STR-containing DNA constructs were then eluted from the beads by adding 20 µl of 0.15 M NaOH and incubating 20 minutes at room temperature with periodic gentle agitation. The NaOH/DNA solution was removed from the beads and neutralized by the addition of 1.3 µl 1.25 M acetic acid and 2.2 µl of 10X TE, pH 7.6. Purification of the unwanted ions proceeded by the addition of 25 µl TE, pH 8.0 to increase the total volume to 50 µl. The total volume was then purified by spun column chromatography using G-50 Sephadex™ (Pharmacia Biotech). Approximately 45-50 µl was recovered from the column, whereby 5 µl could be used for a hot-start PCR amplification. The hot-start PCR setup was as follows:

5 µl 10X Fisher Assay Buffer A (which includes 15 mM $MgCl_2$)

2 µl 25 mM $MgCl_2$ (additional Mg^{2+} ions may help)

4 µl 2.5 mM each dNTP (for 0.2 mM final)

3 µl 30 pmol S61 primer (10 pmol/µl)

5 µl column prepared STR-enriched DNA

30.5 µl ddH_20

49.5 µl Total volume

Filtered mineral oil (38 µl) was added to the top of the PCR sample. A "hot start" procedure was begun by adding 0.5 µl (2.5 U) *Taq* polymerase (FisherBiotech™) after 2 minutes at 94°C. The thermal cycle parameters were:

3 minutes at 94°C

45 seconds at 60°C

1 minutes at 72°C

One cycle was completed then the parameters were changed to:

45 seconds at 94°C

45 seconds at 60°C

45 seconds at 72°C

After 39 cycles it was then linked to:

5 minutes at 72°C

Soak at 4°C

The PCR product was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 3 volumes of -20°C 100% ethanol, followed by centrifugation at 4°C. After a -20°C 70% ethanol wash, the DNA was resuspended in 15 µl of ddH_20. The following was then added: 2 µl 10X *Sau*3AI buffer, 2 µl 10X BSA, and 1 µl *Sau*3AI enzyme (4 U), for a total volume of 20 µl. The mixture was incubated at 37°C for 1 hour and subsequently phenol extacted and ethanol precipitated (same as above). This time the DNA was resuspend in 10 µl ddH_20.

Five microliters of the *Sau*3AI-digested PCR product was ligated to *Bam*HI/alkaline phosphatase treated vector. The ligation mixture was:

5 µl STR-enriched *Sau*3AI-enriched DNA

4 µl *Bam*HI/dephosphorylated pUC18 (or pBluescript® SK-)

6 µl ddH_2O

4 µl 5X ligation buffer (GIBCO BRL®)

1 µl T4 DNA ligase (GIBCO BRL®)

20 µl Total volume

The incubation was allowed to proceed at room temperature 2-4 hours, or overnight at 16°C. A portion of the ligation, 7-10 µl, was then used to transform frozen competent cells (DH5α™). After the heat-shock and out-growth periods, all of the cells were collected by performing a 30 second centifugation at 10,000 x g. Approximately 900 µl of the supernatant was poured off and the remaining volume, 200 µl, was used to resuspend the cells with a mechanical pipetor. All of the cells were then spread-plated onto an LB/ampicillin 50 mg/ml plate, which was subsequently incubated at 37°C overnight.

A typical STR isolation attempt by this method yielded 1-15 white colonies per plate and very few blue colonies. Initial attempts to create replica colony lifts resulted in the loss of some transformants due to the complete transfer of the colony to the membrane. The creation of duplicate master plates prior to performing lifts, by inoculating them sequentially and identically with the toothpick used to pick each isolated white transformant, solved this problem. A Colony/Plaque Screen™ Hybridization Transfer Membrane (NEN™ Life Science Products) was then used on one of the plates and prepared for hybridization with a probe or probes. The membrane lift was hybridized with an end-labeled STR oligonucleotide, the identical sequence of the one or more used to "capture" the microsatellite-containing region. An appropriately-sized piece of high-speed x-ray film (Kodak BioMax™ MR) was then placed on top of the membrane to produce an autoradiograph which revealed the specific colonies, or master plate streaks, which hybridized positively.

Positively hybridized membrane regions indicated which clones on the master plates were likely to encode STRs. These clones were inoculated into 5 ml of LB broth with 50 μg/ml ampicillin and cultured overnight at 37°C. A rapid alkaline lysis plasmid procedure was then performed to provide purified recombinant plasmid templates. The nucleotide sequence of each potential (positive) microsatellite clone was determined using the Sequenase™ version 2.0 (United States Biochemical) DNA sequencing kit. This kit utilizes the dideoxy-mediated chain-termination method of Sanger *et al.* (1978). PCR primers, complementary to the unique flanking sequences of identified STRs, were then designed to yield a product of approximately 200 base pairs. This process was assisted by using the software program Primer3 (Rozen and Skaletsky, 1998). Individual Spoonbill DNA samples, which PCR amplified to give a product of the approximate size, were initially analyzed by 7.5% non-denaturing polyacrylamide gel electrophoresis (PAGE) to ensure the presence of polymorphism. These PCR products were compared in order to determine the total number of alleles present in the population and the degree of heterozygosity for that locus.

Sanger dideoxyribonucleotide DNA sequence analysis of double-stranded templates

Once a clone has been identified as possibly encoding an STR locus, the DNA sequence of the STR flanking regions must be determined in order to design PCR primers which are specific to that locus. The PCR primers can then be used to amplify the locus of interest, if it is relatively short (ideally less than 300 bp), from each Spoonbill genome. The specific nucleotide sequences of cloned DNA templates were determined by the dideoxy-mediated chain-termination method elucidated by Sanger *et al*. (1977). Specific

clones of interest were grown overnight in LB medium, supplemented with the appropriate antibiotic, and microgram quantities (about 60 µg per 5 ml medium) of double-stranded DNA was isolated by the rapid plasmid isolation procedure of Birnboim and Doly,1979 (see above).

Four micrograms of clean (no RNA, etc.) plasmid DNA (7 µl of rapid plasmid preparation) was placed into a 0.6 ml microcentrifuge tube. Twenty-three microliters of sterile ddH_2O was then added to the sample. The DNA was denatured by adding 4 µl of freshly made 2 N NaOH (800 mg in 10 ml ddH_2O) and incubated for 5 minutes at room temperature. One hundred twenty microliters of –20°C 100% ethanol was added and mixed well to ready it for DNA precipitation. Five microliters of 3 M sodium acetate (pH 5.2) was added and mixed well by use of a Fisher Vortex Genie 2™ (Fisher Scientific). The tube was centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was removed carefully without disturbing the pellet (using a drawn-out Pasteur™ pipet) and washed with 120 µl –20°C 70% EtOH. The tube was centrifuged for 5 minutes at 10,000 x g at 4°C, and the supernatant carefully removed. The residual ethanol was removed by placing the tube in a Savant Speed Vac™ vacuum centrifuge for 5 minutes. The DNA pellet was resuspended in 10 µl of the sequencing primer (1 pmol), 5X reaction buffer, and water solution. The primer was annealed to the template by incubating the mixture at 37°C for 45 minutes.

During the primer/template incubation, sets of "termination tubes" were prepared for each sample. Each set consisted of four 0.5 ml microcentrifuge tubes, labeled with the clone number and color coded, orange, green, blue, yellow, to represent G, A, T or C, respectively.

2.5 µl of ddGTP termination mix added to the G tubes. (orange)

2.5 µl of ddATP termination mix added to the A tubes. (green)

2.5 µl of ddTTP termination mix added to the T tubes. (blue)

2.5 µl of ddCTP termination mix added to the C tubes. (yellow)

The labeling solution was prepared for a set of 12 templates by combining the following in a 0.5 ml microcentrifuge tube.

DTT (0.1M)	13.0 µl
distilled water	22.8 µl
dGTP labeling mix	5.2 µl
α-^{35} S-dATP	6.5 µl
Total volume	47.5 µl

After the 45 minute incubation, 3.5 µl of the labeling solution was added to each of the annealed primer/template tubes. The Sequenase™ (Version 2.0) enzyme was diluted 8-fold in a 0.5 ml microcentrifuge tube using the Sequenase™ dilution buffer as outlined below. The quantities shown are sufficient for sequencing 12 samples.

Sequenase™ dilution buffer	21.5 µl
pyrophosphatase	1.7 µl
Sequenase™	3.3 µl
Total volume	26.5 µl

The diluted Sequenase™ was added to each of the tubes containing annealed primer/template mixtures using a timed protocol. Each template received 2 µl diluted Sequenase™ and was allowed to incubate at room temperature for 4 minutes. A 3.5 µl aliquot of each template/ Sequenase™ mixture was then immediately added, and mixed

well by repeated pipetting, to each of the four (G/A/T/C) termination tubes, which were pre-warmed to 37°C in a water bath. The termination tubes were incubated at 37°C for 20 minutes to allow the dideoxyribonucleotides to randomly incorporate into the growing DNA strands. Four microliters of a formamide-based "stop" solution and loading dye was added to each of the G/A/T/C tubes. Each tube was briefly centrifuged and then stored at -20°C until ready for use.

Denaturing polyacrylamide sequencing gels

A properly poured sequencing gel is one of the most critical steps of the entire sequencing process. A failure during this procedure would waste hours, if not days, of a continuous process. The cassette assembly was performed with extreme cleanliness, to prevent band disruption from dust fragments, and carefully in order to prevent leakage during the pouring process. This assembly procedure was adapted from Sanger and Coulson, 1978.

The assembly process began with thoroughly cleaning two glass plates (52 x 41 x 0.6 cm). These were laid on four large stoppers (#15 is an appropriate size) and cleaned thoroughly with glass cleaner, then with 95% ethanol and allowed to dry completely. Both plates were coated on one side with a thin layer of 5% dichlorodimethylsilane dissolved in heptane. Two to three coats were necessary to insure that the plates were fully covered. One plate was only coated once in an effort to ensure that the gel would not "float out" of the cassette when electrophoresed. The plates were allowed to completely dry (approximately 5 minutes), and gently wiped with Kimwipes® before

completing the cassette assembly. The silinating was performed in a vented hood to prevent inhalation of the potential carcinogen.

One of the glass plates was positioned on top of the other so that they were offset by about 13 mm (1/2 inch) lengthwise, with the siliconized sides facing each other. The plates were separated on the two long sides by 51 cm x 13 mm x 0.25 mm Delrin® spacers. On each side, 4-5 medium-sized binding clips were used to hold the plates together. A third spacer (about 45 cm x 7 mm x 0.25 mm) inserted in the bottom of the cassette (the bottom of the cassette later became the top of the gel when it was placed into an electrophoresis stand) was clamped with 4 large binding clips. Two short pieces (3 cm x 13 mm x 0.25 mm) of Delrin® were inserted into the top of the cassette and clamped into place with large binding clips. They were inserted only 0.5 cm in an effort to not leave an air bubble when removed for gel electrophoresis.

A "funnel reservoir" was constructed across approximately one-third of the top of the cassette with warmed (kept at 37°C) Plastocene® (or modeling clay). It was molded to be slightly higher at the outside edge and tapered off as it approached the center of the cassette. The funnel reduced spillage of acrylamide and helped avoid the formation of air bubbles as the solution entered the cassette. A 6% polyacrylamide, 7 M urea, sequencing gel solution was prepared as follows:

42.4 g urea

20 ml of 30% acrylamide/1% bisacrylamide

10 ml 10X Sequencing Gel Buffer: 100.0 mM Tris base

12.0 mM boric acid

1.0 mM EDTA

X ml ddH_20 (bring up to volume)

100 ml Total volume

It was stirred thoroughly before bringing up to the final volume of 100 ml. Undissolved particles were removed from the solution by filtering it through a Buchner funnel containing a piece of qualitative P5 filter paper (Fisherbrand®). The filtrate was collected in a 250 ml Erlenmeyer vacuum filtration flask to which 0.12 g solid ammonium persulfate was added. The solution was degassed *in vacuo* by placing a rubber stopper on top of the flask until all dissolved gasses were removed (only 30 to 45 seconds). Twenty microliters of TEMED (*N,N,N',N'*-tetramethylethylenediamine) catalyst were added and the flask briefly swirled in a fashion as to not introduce bubbles while mixing the solution well. The solution was slowly poured into the funnel of the vertical gel cassette, which was supported at a 45° angle left to right, and allowed to track smoothly around the perimeter of the cassette and, finally, to fill it as it was slowly laid horizontally. The gels polymerized within fifteen to thirty minutes and could be used for electrophoresis within 1.5-2 hours.

The short spacers and the 45 cm spacer were removed and the cassette clamped to a vertical electrophoresis chamber. Each chamber reservoir was filled with 1 liter of 1X Sequencing Buffer. Air bubbles were removed from the well space and the bottom of the gel by Pasteur™ pipets and bulbs. The power leads were connected and the gel was pre-electrophoresed at 50 W, constant power, for 1 hour. The well region and bottom of the cassette were then again rinsed well with Pasteur™ pipets to remove bubbles that had formed and excess urea which diffused from the gel. Shark's tooth well-forming comb

units were inserted into the top well area in a uniform manner and with each tip barely touching the gel.

The dideoxyribonucleotide-treated samples were heated to 90°C for two minutes before being immediately transferred to an ice/water bath. This eliminated any intra-strand base pairing that might have influenced the migration rate of the samples. With the power turned off, a two microliter aliquot of each sample was loaded onto the gel using a 10 µl Hamilton® syringe (32 G needle). Each termination tube sample was placed into a separate well in the order G, A, T, and C. One lane was skipped between each 4 sample set. Once all samples were loaded, the power was set to 50 W and the gel allowed to electrophorese for 3.5 hours. This typically allowed the xylene cyanol to migrate approximately 45 cm from the top of the gel and corresponded to a fragment of approximately 70 bases from the +1 primer binding site migrating to the bottom of the gel.

When the gel had electrophoresed for the proper time, the power was turned off and the buffer reservoirs drained by siphonage. The cassette was then removed and placed flat onto the bench top. The clips and spacers were removed and a thin spatula inserted into one corner. Slowly and with gentle pressure, a spatula was used to wedge apart the plates. On most occasions the gel would stick entirely to the less siliconized plate and be ready for immediate transfer to filter paper. A 14" x 17" piece of thick filter paper was lined up with the bottom of the gel and centered side to side (making sure it covered all gel lanes utilized), then placed on top of the gel. The paper was firmly pressed against the gel to remove any air bubble using the flat edge of a razor blade as a scraper against the paper. Any excess gel protruding from the filter paper was cleanly cut

away with the razor blade. The plate was then inverted and the gel slowly released to the countertop. A layer of plastic wrap was then added to protect the gel during the drying process. Excess plastic wrap was trimmed away and the protected gel on filter paper was transferred to a gel drying apparatus. The gel was dried for 1 hour at 75°C and the plastic wrap removed. During humid conditions the gel was leached of the hydroscopic urea by soaking it in a 10% acetic acid, 12% methanol solution for 15 minutes. The gel was then placed on the bench and again covered with plastic wrap. After the excess plastic was trimmed, the gel was dried a second time at 75°C for 90 minutes. Once again, the plastic wrap was removed after drying.

Autoradiography was performed on the sequencing gel using Kodak's 14" x 17" BioMax™ MR high speed autoradiography film, sandwiched in an aluminum six-point clamping cassette. Routinely the exposure time was 12-14 hours, but would be continued longer as the isotope proceeded through its half-life. The films were processed by an automated developer (ALL-PRO 100), which contained the developer, fixer, water wash and dryer combined.

Vertical polyacrylamide gel electrophoresis with DNA detection by ethidium bromide, autoradiography or fluorescent scanning

Separation of small, less than 500 bp, double-stranded DNAs created by PCR amplification was routinely performed using high resolution agarose such as NuSieve®3:1 (FMC®). When the amplified products were less than 200 bp and the differences between samples were as little as 2-4 bases, the resolving capability of the agarose electrophoresis system was not adequate. Polyacrylamide gel electrophoresis

was optimized to allow differentiation of samples which were separated by only a few nucleotides. Since PCR amplification of polymorphic STR (microsatellite) loci often resulted in 2 alleles of less than 200 bp, with only a 2-4 bp difference in size, the procedures to assemble polyacryamide gel cassettes are outlined below.

Many different cassette sizes and acrylamide concentrations can be utilized for polyacrylamide gels, but only two types were extensively used in these experiments. The very short, 20 cm x 20 cm, cassette was used to verify the presence of suspected polymorphic PCR products. Two glass plates, one 20 cm x 20 cm and the other 20 cm x 22 cm, were thoroughly cleaned with Windex® glass cleaner and 95% ethanol. Two spacers, 1.5 mm thick and 1.5 cm wide, were then lightly coated with vacuum grease on the outer half, top and bottom sides, and placed on either side of one of the plates. The other plate was then carefully placed on top of the spacers with clamps added to hold the plates together. The cassette assembly was placed on a flat surface, vertically, and gentle pressure applied to level the plates and spacers relative to one another, which helped prevent leakage during the addition of the acrylamide solution. The cassette was then placed onto a neoprene spacer, which had a very light layer of vacuum grease, and clamps were added to keep it immobile. Non-denaturing acrylamide gel solution was then prepared.

A sixteen milliliter volume of 37:1 acrylamide/bis-acrylamide was added to a graduated cylinder. Eight milliliters of 10X non-denaturing buffer (500 mM Tris, pH set to 8.3 with solid boric acid, 10.0 mM EDTA, ethylenediaminetetraacetate, disodium form) was mixed with the acrylamide/bis-acrylamide solution. Distilled, de-ionized water (56 ml) was used to bring the volume up to 80 ml, giving a 7.5% polyacrylamide

solution. Ammonium persulfate, 0.1 g, was added to the solution in a vacuum flask and mixed by swirling. A vacuum was applied (only 30-45 seconds) to de-gas the mixture. A polymerization catalyst, 5 µl TEMED (*N,N,N',N'*-tetramethylethylenediamine), was added and gently swirled before the solution was poured into the cassette. The comb was inserted after it was determined no bubbles were present in the assembly, which would inhibit polymerization, and three large clamps were used to sandwich the comb, like a spacer, to ensures uniform gel thickness. The cassette was left in a horizontal position to help prevent leakage and help promote uniform gel thickness. Polymerization was considered complete after 1.5 hours. The top of the cassette was wrapped with plastic wrap if it was to be allowed to sit overnight and the gel run the next day.

The cassette was then placed into a vertical electrophoresis chamber and 1 liter of 1X non-denaturing buffer was equally dispensed into the upper and lower reservoirs. The gel was pre-electrophoresed for 10 minutes at 200. Each PCR amplified sample (8 of 25 µl total) was diluted with 5X loading buffer (25% glycerol, 0.5% SDS, 0.1% bromophenol blue, 0.1% xylene cyanol FF, 50 mM EDTA) and placed in a washed well of the gel. The pBR322 *Hin*fI-digested DNA was placed in two of the 18 wells as size standards. The gel was then electrophoresed at a constant 200 V for 5 hours.

The buffer was drained from the reservoirs by inverting the unit over a sink. The gel cassette was then removed and disassembled to leave the gel on one plate, which was then submerged in an EtBr solution (0.5 µg/ml) for 15 minutes. The plate/gel was then transferred to a destaining water bath where the gel was floated off the plate with gentle coaxing. After 15 minutes of destaining, the gel was lifted by using a 35 cm x 60 cm piece of flexible nylon window screening and transferred to a UV transilluminator. The

electrophoretic profile on DNAs was digitally stored by using the Kodak™ EDAS 120 digital photography system.

The use of longer plates (32 cm x 20 cm and 34 cm x 20 cm), thinner spacers (0.4 mm), and a 19:1 acrylamide:bis-acrylamide solution allowed the gel electrophoresis to proceed much faster and resulted in improved resolution of PCR products. The separation of PCR products on this gel can be visualized by numerous methods, including silver staining (not performed), autoradiography, or fluorescent scanning. The preferred method of gel analysis was the use of a fluorescent scanner, such as the Hitachi FMBIO® II Fluorescent Scanner, access to which was generously provided by Dr. Arthur Eisenberg's DNA Identity Laboratory, University of North Texas Health Science Center, Fort Worth, Texas.

Assembly of the cassette differed depending upon the expected means of DNA detection. If the gel was to be analyzed by silver staining or autoradiography, after gel electrophoresis it was necessary to dismantle the cassette and work with the gel on one plate. To ensure the proper adhesion of the gel to only one plate, diluted bind silane (γ-methacryloxypropyltri-methoxysilane) was used. It was prepared fresh each time by adding 1.5 µl bind silane and 2.5 µl acetic acid to 496 µl 100% ethanol in a 1.5 ml microcentrifuge tube. The solution was spread liberally and completely onto the shorter plate (32 cm x 32 cm) while wearing doubled gloves and using Kimwipes®. After 5 minutes, the excess bind silane was removed by wiping the plate three times with 100% ethanol. The top layer of gloves was removed before spreading 5% dichlorodimethylsilane dissolved in heptane onto the other plate using Kimwipes®.

Assembly of the plates proceeded with care to not allow them to touch each other. The slightest contact could have transferred bind silane to the silinated plate and caused the gel to adhere to both plates in that region, causing it to rip the gel during disassembly. Two 32 cm x 1.5 cm spacers (0.4 mm thick) were then placed on the outer edges of one plate. Since a special rubber boot (GIBCO®) was not available to prevent leakage of acrylamide during pouring, the outer halves of each spacer were coated with a thin layer of vacuum grease. The second plate was added and clamps were placed to hold the plates and spacers together. Care was taken to not allow any vacuum grease to reach the interior of the cassette, since this would inhibit gel polymerization. The bottom of the cassette was placed on a neoprene spacer, to prevent gel leakage during pouring, and held in place with clamps.

A 4% acrylamide gel was prepared by mixing 18.9 g urea, 4.5 ml 5X TBE, 4.5 ml 40% (19:1) acrylamide:bis-acrylamide solution, and ddH_2O up to an approximate volume of 40 ml. Once the urea totally dissolved into the solution the volume was set to exactly 45 ml with ddH_2O. The solution was then passed through P5 filter paper before adding to a 50 ml hypodermic syringe. Thirty microliters of TEMED and 300 µl 10% ammonium persulfate (kept as an frozen aliquot) were then added to the solution and mixed gently. The acrylamide was carefully added to the corner of the well end of the plate while the assembly was almost horizontal. Once the solution filled the assembly, a square-toothed comb was added and held in place with 3 large clips. Polymerization was allowed to proceed for a least 1 hour before the cassette would be clamped into a GIBCO BRL® SA Electrophoresis Apparatus using 0.5X TBE buffer in the tanks. The custom made plates received from Dr. Arthur Eisenberg's DNA Identity laboratory (University of North

Texas Health Science Center, Fort Worth, Texas) were thinner than the commercially supplied plates, so one or two extra plates were added to the outside of the cassette to assist in the clamping process.

Each well, and the bottom of the assembly, was rinsed 3 times with a 19-guage needle and syringe to remove any trapped air bubbles. The current practice was to not pre-electrophorese the chamber before adding the samples. The PCR products were mixed 50:50 with a bromophenol blue loading solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue) by adding 4 µl PCR reaction with 4 µl loading solution. The mixtures were denatured at 95°C for 2 minutes, then placed directly on ice. Three microliters of each sample was loaded immediately and as quickly as possible to prevent re-annealing of the PCR products. Once the samples were loaded, the gel electrophoresed at 50 W for 1 hour. After the gel electrophoresis was complete, the cassette was removed and gently cleaned with water to remove any TBE buffer. Image processing by the FMBIO® II Fluorescent Scanner required 5 to 10 minutes.

If the PCR products were isotopically-labeled, the glass plates were separated and the plate with the attached gel was placed in a shallow tray with 10% acetic acid and 12% methanol. Urea was leached out of the gel over a period of 20 minutes. The plate with gel was lifted from the solution and placed on a gel drying slab set to 60°C, with a fan set on low speed directed across it (no vacuum). It usually required about an hour to thoroughly dry the gel. A thin piece of transparency acetate was placed over the entire gel and plate and secured tightly with Scotch® tape. With the gel side up, it was placed in an x-ray film cassette with a piece of Kodak BioMax™ MR film. Autoradiography proceeded for 6 to 24 hours.

Creation of a lambda ZAP Express® small-insert genomic library

Construction of a small-insert Spoonbill genomic library in a lambda vector began with the purchase of the ZAP Express® Undigested Vector Kit (Stratagene®) and MaxPlax™ Lambda Packaging Extracts (EPICENTRE®). A useful property of the ZAP Express® vector is its ability to accommodate DNA inserts from 0 to 12 kb in length. Since an average microsatellite region contains only 30 to 100 bp, and PCR primers are designed from the 20-40 bp flanking on each side of the microsatellite, restriction endonuclease digested DNA inserts of approximately 400 to 800 bp were selected to be inserted into the Lambda library. Two Spoonbill genomic DNA libraries were created with this vector. One library was a *Sau*3AI digestion of genomic DNA ligated into a *Bam*HI-cut vector, and the second was a *Tsp*509I digestion of genomic DNA ligated into an *Eco*RI-cut vector.

Five micrograms of Spoonbill genomic DNA was digested with either *Sau*3AI or *Tsp*509I and electrophoresed at 30 V for 1 hour using 0.8% LMP agarose in 1X TAE. Each DNA smear was excised in the range of 400-800 bp and then purified by the use of the Prep-A-Gene DNA Purification Kit (Bio-Rad). The DNA was eluted in a total volume of 50 µl ddH_20. Control ligations were preformed with 0.5 µl to 3 µl aliquots of the DNA solution and appropriately digested pBluescript II SK(-).

The lambda DNA was prepared by digestion with either *Bam*HI or *Eco*RI. The digested DNA was then treated with calf intestinal alkaline phosphatase (CIP). Ligation of the 3 µl size-selected DNA (approximately 0.2 µg) to 1 µl (1 µg) lambda arms proceeded with the use of 0.5 µl 10X Ligase Buffer (500 mM Tris-HCl, pH 7.5, 70 mM

$MgCl_2$, 10 mM dithiothreitol), 0.5 µl 10 mM ATP, and 0.5 µl (1 U/µl) T4 DNA Ligase (GIBCO BRL®). The ligation mixture was incubated at 13.5°C for 21 hours.

The cloned DNA (lambda arms with ligated DNA inserts) was packaged by use of the MaxPlax™ Lambda Packaging Extract. A 25 µl volume of packaging extract was removed from the –70°C freezer, thawed at room temperature and mixed with the cloned DNA (5.5 µl) in a 0.6 ml microcentrifuge tube. The tube was then incubated at 30°C for 90 minutes. An additional 25 µl thawed extract was added to the reaction tube and mixed well without introducing bubbles. The tube was again incubated at 30°C for 90 minutes. Five hundred microliters of phage dilution buffer (10 mM Tris-HCl, pH 8.3, 100 mM NaCl, 10 mM $MgCl_2$) was then added and mixed by gentle vortexing. Twenty-five microliters of chloroform was added, mixed well by gentle vortexing, and the tube was stored at 4°C.

Each Spoonbill genomic ZAP Express® library was titered and tested for background (lambda DNA with no inserts) by first mixing 100 µl of the diluted (10^{-2}) packaged phage with 200 µl XL1-Blue MRF' (at an OD_{600} of 0.5) and incubating the mixture at 37°C for 15 minutes with periodic gentle shaking to allow the phage to attach to the cells. After this attachment period, 15 µl of 0.5 M IPTG (in ddH_20), 50 µl of 250 mg/ml X-gal (in dimethylformamide) and 3 ml of 48°C NZY top agar (5 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 5 g yeast extract, 10 g casein hydrolysate, deionized H_20 to 1 liter, pH 7.5, and 0.7% (w/v) agarose) were added to the phage/cell mixture and immediately poured onto a pre-warmed NZY agar plate. The plates were allowed to solidify for 10-15 minutes and were then inverted and placed into an incubator at 37°C for 12-16 hours. The recombinant lambda ZAP Express® plaques appeared as cleared zones amongst a

bacterial lawn. The background (non-recombinant) plaques were identified by their blue color.

When a specific plaque of interest was identified, it was removed from its agar plate by the use of a borosilicate transfer pipet and a rubber bulb. The plaque, with an agar core, was then transferred into a microcentrifuge tube which contained 500 µl of SM buffer (5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 50 ml 1 M Tris-HCl, pH 7.5, 5 ml 2% w/v gelatin in 1 liter ddH_2O, autoclaved) and 20 µl of fresh chloroform. This tube was then stored at 4°C for up to 6 months.

If the lambda clone needed to be further characterized, such as determination of the DNA sequence, an *in vivo* excision procedure was used to release a functional copy of the pBK-CMV phagemid clone from the ZAP Express® vector (Stratagene, 1999). Once the phagemid clone was created within bacteria cells, it was processed through a rapid plasmid isolation procedure and then the DNA sequence was determined.

The phagemid excision procedure was begun by growing a culture of XL1-Blue MRF' cells in LB medium, supplemented with 0.2% (w/v) maltose and 10 mM MgSO4, overnight at 30°C in a gyrotary incubator and shaker at 250 rpm. The clone of interest was stored in SM buffer and chloroform (see above) for a minimum of 1 hour at 23°C. The XL1-Blue MRF' cells were grown to an OD_{600} of approximately 1.0. Then a mixture of 200 µl MRF' cells, 250 µl of phage stock, and 1 µl of ExAssist™ helper phage (>1 x 10^6 pfu/µl) were placed in a 15 ml polypropylene tube. These reagents were allowed to incubate at 37°C for 15 minutes with periodic gentle shaking. A volume of 3 ml NZY broth was then added to this mixture and allowed to incubate at 37°C in a gyrotary incubator and shaker at 250 rpm for 16-20 hours. The polypropylene tube and

its contents were then heated to 65°C for 20 minutes. All of the cellular debris was pelleted by a centrifugation at 2,200 rpm for 15 minutes in a Sorvall® T6000B with 15 ml adapters. The supernatant was decanted to a sterile 15 ml polypropylene tube and stored at 4°C for 2 months.

Since the supernatant in the polypropylene tube contained the excised pBK-CMV phagmid clone packaged as filamentous phage particles, it was transferred to host cells (XLOLR) and grown as a circular plasmid. This procedure was begun by growing a culture of XLOLR cells overnight in NZY broth at 30°C in a gyrotary incubator and shaker at 250 rpm. The XLOLR cells were grown to an OD_{600} of approximately 1.0. Then a mixture of 200 µl XLOLR cells and 100 µl of phage supernatant were placed in a 1.5 ml microcentrifuge tube. These cells and the phagemid clone were allowed to incubate at 37°C for 15 minutes with periodic gentle shaking. A volume of 300 µl NZY broth was then added to this mixture and allowed to incubate at 37°C in a gyrotary incubator and shaker at 250 rpm for 45 minutes. A portion of the cell mixture, 200 µl, was spread-plated onto an LB-kanamycin (50 µg/ml) agar plate. The plate was incubated overnight at 37°C. Thousands of colonies were present on an agar plate from successfully excised clones. One of these colonies was then chosen for a plasmid isolation procedure and finally, the DNA sequence was determined. The primers T3 and T7 should be used to sequence the pBluescript™ vector. They bind closer to the MCS and allow for longer reads into the DNA inserts.

CHAPTER III

RESULTS AND DISCUSSION

The creation of a DNA-based identity test is contingent upon the isolation and discovery of polymorphic loci within the genome under study. One method by which these loci can be identified is the direct sequencing of an organism's entire genome, an approach which is occurring increasingly because of the advances in high through-put DNA sequencing machines. However, since genome sequencing is very expensive and funding for such a project is often dependent upon finding cures or treatments for specific deleterious genes, the Spoonbill and other wild bird genome sequences are not likely to be determined for many years and most likely decades. The genome of the domestic chicken (*Gallus gallus*) is an exception due to its agricultural value, and so efforts to better understand its genome and to improve its husbandry are well advanced. Our efforts to create an identity test for the Roseate Spoonbill can benefit from the studies of the domestic chicken genome, particularly when analyzing specific homologous loci.

Multilocus probing

This project's initial work was to develop a DNA-based identity test for Roseate Spoonbills utilized the multilocus probe, pV47-2, which has been used in previous studies of a variety of mammalian and avian species (Longmire *et al*., 1990), and a microsatellite probe, $(CA)_n$, which also acts as a multilocus probe where the number of loci bound depends upon the frequency of $(CA)_n$ microsatellites in the species under study. Spoonbill genomic DNA was isolated from 16 whole blood samples provided by the Fort Worth Zoo (See Table IV, page 128). RFLP analyses of DNAs from these

individuals were performed using a battery of restriction endonucleases (e.g. *Hin*fI, *Hae*III, *Pvu*II, *Rsa*I, *Hin*dIII, *Hin*cII, *Bgl*I, and *Bgl*II) and the two aforementioned multilocus probes, pV47-2 and (CA)n. The majority of the banding patterns observed on the autoradiograph of the hybridized genomic DNA showed the test population to be non-polymorphic at the $(CA)_n$ loci with most enzymes (Fig. 4). However, the fragment pattern of two restriction endonucleases, *Hae*III and *Bgl*II, did show some polymorphism at pV47-2 loci (Figs. 5 and 6). The combination of *Bgl*II-digestion of Spoonbill genomic DNA and the pV47-2 probe yielded the most informative polymorphic banding patterns. However, since the exhibited levels of polymorphism and heterozygosity were not sufficient for conclusive DNA typing of individuals from a large population, it was determined that other probes, specifically single-locus probes for known polymorphic loci, would be needed.

Search for polymorphic MHC loci

The polymorphic nature of MHC loci has been utilized as a resource to DNA profile humans for a number of years (Bunce *et al*., 1993). Several chicken MHC loci have been identified and characterized, and it was therefore considered likely that these would be useful as probes for MHC loci in other avian species such as the Spoonbill. The chicken class II (*B-L*-encoding) molecules are composed of two distinct non-covalently linked polypeptide chains. The chains are named α and β (Crone and Simonsen, 1987), and like their mammalian counterparts have been shown to be polymorphic primarily in the β chain (Zoorob *et al*., 1993).

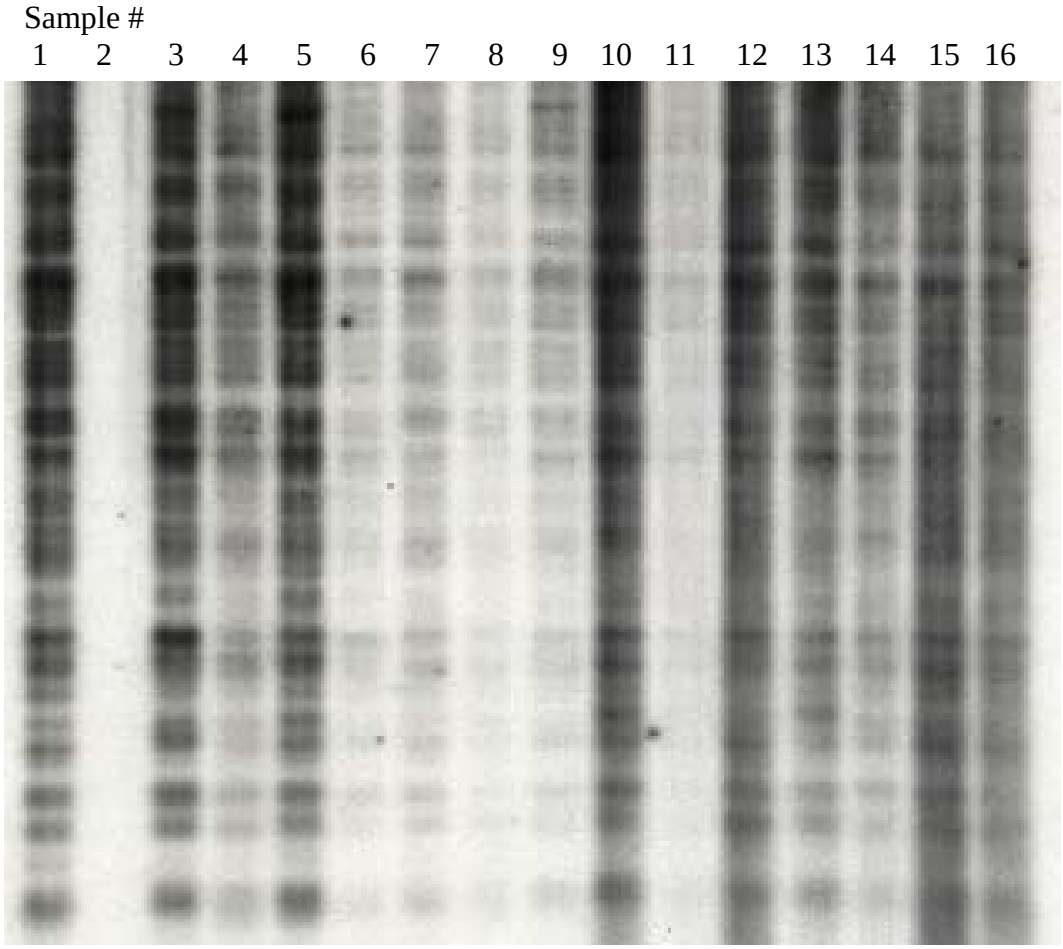

Figure 4. **Autoradiograph of Spoonbill genomic DNA digested with *Bgl*II and hybridized with the $(CA)_{12}$ microsatellite probe.** A randomly selected collection of 16 reference Spoonbill DNA samples (Table IV) were digested with *Bgl*II, then subsequently electrophoresed and hybridized with a γ-^{32}P-labeled $(CA)_{12}$ oligonucleotide in an effort to determine whether polymorphic CA-microsatellite loci were present in the Spoonbill genome. The same reference samples were used in Figs. 5, 6 and 7.

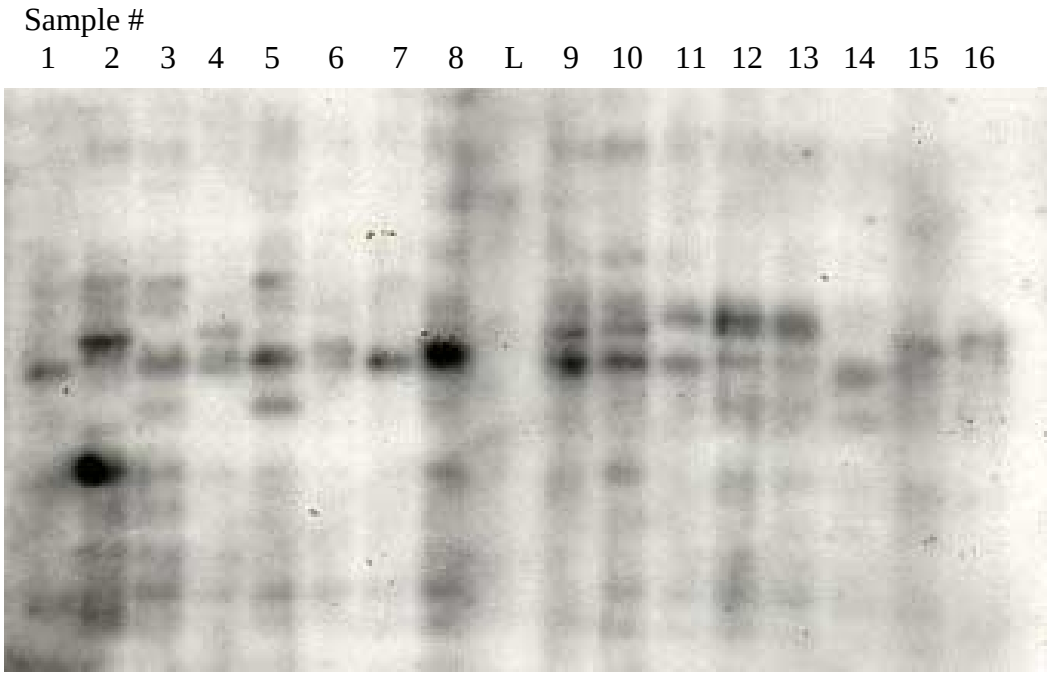

Figure 5. **Autoradiograph of *Hae*III-cut Spoonbill genomic DNA hybridized with the multilocus probe pV47-2.** The 16 reference samples (Table IV) were the same as those used with the microsatellite probe $(CA)_{12}$, Fig. 4, and were also used in Figs. 6 & 7. The letter "L" refers to the DNA size standard (not hybridized): lambda DNA digested with *Hin*dIII.

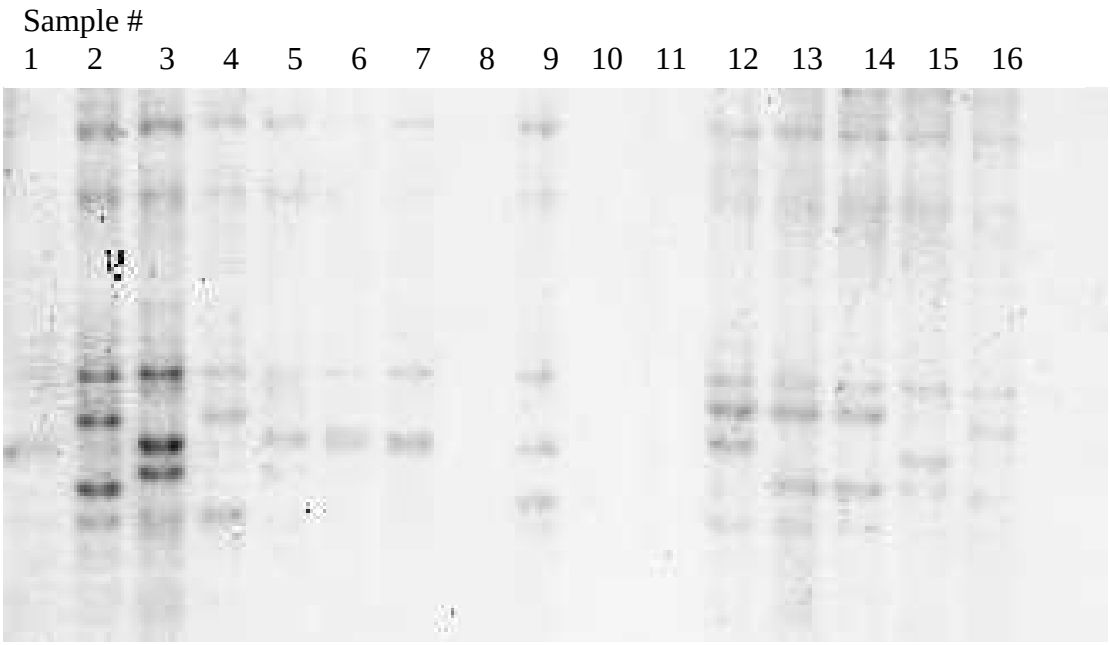

Figure 6. **Autoradiograph of Spoonbill genomic DNA digested with *Bgl*II and hybridized with the multilocus probe pV47-2.** The 16 reference samples (Table IV) are the same as in Figs. 4, 5 and 7. A size standard was not loaded on this agarose gel.

β chain probe

The 2.3 kbp chicken MHC β chain genomic clone CCII-7-1 (Xu *et al*., 1989), used as a probe to detect two or three restriction fragments (depending upon the enzyme used) within chicken sperm DNA (Chen *et al*., 1997), was acquired from Dr. Susan Lamont and used to probe *Bgl*II-cut Spoonbill genomic DNA samples for the possible existence of restriction fragment length polymorphisms (RFLP). The hybridization temperature was only moderately stringent (60°C) to allow the chicken probe to bind to homologous Spoonbill genomic regions. The banding pattern obtained was only very weakly discernable, even after low stringency washes (two at room temperature using 2.5X SSC, 0.1% SDS), and appeared to be effectively non-polymorphic (Fig. 7). Since the pattern obtained exhibited no obvious polymorphism in the test individuals, and it was not clear that the chicken probe was specifically hybridizing to the homologous Spoonbill locus, RFLP studies of this locus were suspended.

β chain PCR amplifications

PCR primer sets were designed based upon the known nucleotide sequences of chicken MHC β1 exons, specifically B-G cDNA sequences (Kaufman *et al*., 1989), in an effort to PCR amplify and clone homologous regions of Spoonbill DNA which might exhibit single nucleotide polymorphisms (SNPs). The primers were chosen to be complementary to the most conserved portions of sequences flanking known polymorphic regions. Primer set #1 (S933-1 and S933-2), designed from clone G1 (Kaufman *et al*., 1989), was expected to give a PCR product of 443 base pairs.

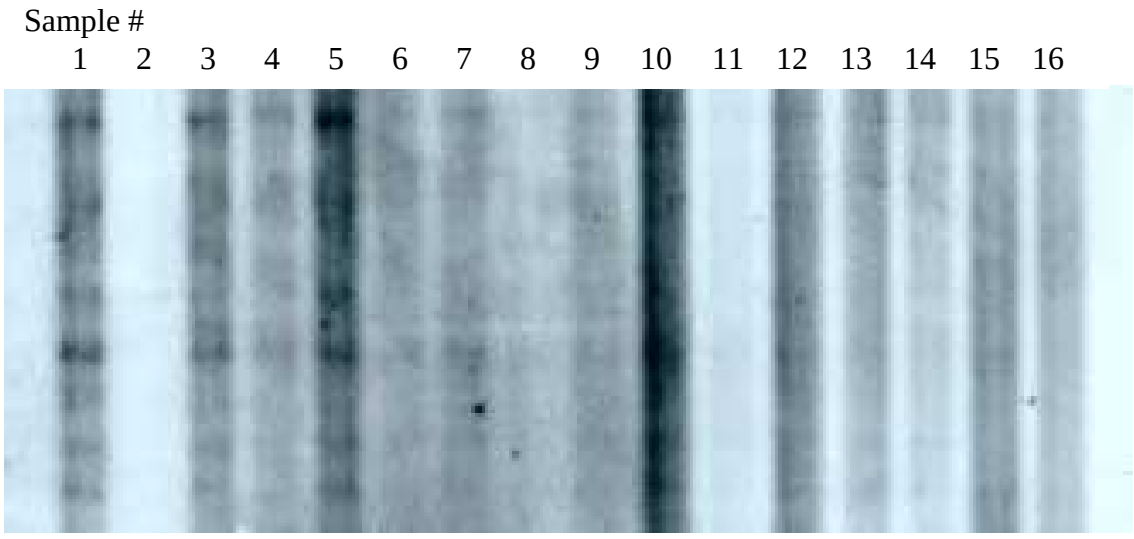

Figure 7. **Autoradiograph of Spoonbill genomic DNA cut with *Bgl*II and hybridized with the chicken MHC clone CCII-7-1.** The same 16 samples were used in Figs. 4, 5 and 6. Perhaps because of the moderately stringent hybridization and wash conditions (see text), the banding pattern (non-polymorphic) was barely discernable.

Primer S933-1: 5'-AGGGTCTCCCCATGGCATCA-3'

Primer S933-2: 3'-CAATTCTCCACTTCGTCTGT-3'

The PCR amplification of Spoonbill genomic DNA routinely consisted of the following mixture:

10 µl	genomic DNA (3-4 ng/µl)
5 µl	10X PCR Buffer A, includes 15 mM $MgCl_2$ (FisherBiotech™)
7 µl	S933-1 (5 µM)
7 µl	S933-2 (5 µM)
3 µl	dNTPs (2.5 mM each of the four dNTPs)
1 µl	*Taq* DNA polymerase (5 U/µl) (FisherBiotech™)
17 µl	ddH_2O
50 µl	Total volume

The DNA Thermal Cycler (Perkin-Elmer Cetus Model No. N801-0180) parameters were programmed to begin with 3 minutes at 94°C. This was followed by 30 cylces of 94°C for 1 minute, 59°C for 30 seconds, and 72°C for 2 minutes. The temperature was then held at 72° C for 2 minutes followed by a constant hold at 6°C. The PCR products (12 µl each sample) produced smears from 1,500 bp to 500 bp when electrophoreticly analyzed on 1% agarose gels, with a band around 1,000 bp. Primer set #2 (T692-1 and T692-2) was designed to amplify the entire cDNA clone G3 (1,073 bp).

Primer T692-1: 5'-AGTGCTGAAAAAAGACAGTG-3'

Primer T692-2: 5'-CCATCAGCAGGTATTCTTTA-3'

PCR amplification of Spoonbill genomic DNA again yielded only a smear of DNA when analyzed on a 2% agarose gel.

Many additional attempts were then made to PCR amplify this region in Spoonbill genomic DNA samples. Modifications to the standard PCR setup included: (1) nucleotide concentration increased [from 150 µM to 200 µM in the final concentration], (2) DNA template concentration increased [from 30 to 100 ng total], (3) DNA template concentration decreased [from 30 to 10 ng total], (4) increased the amount of *Taq* polymerase to 7.5 units [from 5 U], (5) the use of an alternate polymerase, Vent™ (5 U) from New England Biolabs, (5) and alteration of the primer concentrations [from 0.7 µM to 0.4 µM in the final concentration]. The use of the high-fidelity (5-15-fold higher than *Taq* DNA polymerase) enzyme, Vent™ polymerase, yielded a faint 6.5 kb fragment. The PCR amplification of different Spoonbill samples was also tried, as well as adjusting the cycle parameters of the DNA Thermal Cycler. The annealing temperature was varied from 59°C to 65°C in 1° increments and the annealing time was increased to 45 seconds.

The optimized conditions [0.4 µM each primer, 50 ng genomic DNA, 150 µM each dNTP, 5 U *Taq* DNA polymerase, 62°C annealing temperature for 30 seconds] for PCR amplification of Spoonbill genomic DNA with the second primer set (T-primers) allowed the production of two bands of approximately 1,000 bp each and a third band of about 1,500 bp. However, attempts to ligate these PCR products into *Sma*I-digested pUC18 (Yanisch-Perron *et al*., 1985), alkaline phosphatase treated or not, were not successful. To improve the ligation efficiency, the PCR product was purified by use of the Spinbind® (FMC BioProducts) kit. This DNA recovery kit was designed to isolate larger fragments from a reaction tube or an agarose gel. Most importantly, it facilitated the removal of primer dimers from a PCR amplification. Primer dimers readily ligate into a vector and seriously limit the cloning efficiency. However, rapid plasmid

preparations of ligated and transformed DNA showed no signs of an inserted fragment into the multiple cloning site (MCS) of vector pUC18.

From this experience it appeared that ligatable ends were not created by *Taq* DNA polymerase. To ensure that PCR amplification products could be ligated to vectors in the future, primer set #2 was redesigned with convenient restriction endonuclease cleavage sites at the 5'-ends. Primer set #3 (SB-3A and SB-3B) was similar to set #2 but with *Eco*RI sites (underlined) on the 5'-ends of each.

Primer SB-3A: 5'-CCGAATTCAGTGCTGAAAAAAGACAGTG-3'

Primer SB-3B: 5'-CCGAATTCCATCAGCAGGTATTCTTTA-3'

Gel analysis of the PCR amplification products yielded a 1,000 bp band (inside a smear) with a 1,500 bp band and the ever-present primer dimers. Primer SB-4A was created to replace the *Eco*RI site in SB-3A with an *Xba*I site (underlined) and to move the primer downstream 20 bp.

Primer SB-4A: 5'-GGTCTAGATGGGTTATTGGCTTTGGAGA-3'

The PCR product produced from SB-4A and SB-3B included a fragment of approximately 900 bp with a dark smear and primer dimers. Further optimization of the PCR conditions [7 cycles of the annealing temperature set at 57°C for 25 seconds followed by 25 cycles of the annealing temperature set at 62°C for 30 seconds] resulted in two bands; 600 and 800 bp with a faint smear. These products were cut with *Xba*I and *Eco*RI then purified with the Spinbind® (FMC) kit. Ligations and transformations revealed no DNA inserts within the pUC18 MCS.

Primer set #5 was created after careful analysis of cDNA clones G3 and G7 (which are 80% homologous). This primer set was expected to PCR amplify a 205 bp

segment, which did not contain a repeat sequence, nor an intron in any known clone of this segment for an avian species. The *Eco*RI digestion sites are underlined.

Primer SB-5A: 5'-GGGGATCCAATAACTGCACAGCATACAAAA-3'

Primer SB-5B: 5'-GGGGATCCTCTGTCTTCCTCCCCTTGCCTT-3'

After PCR amplification of Spoonbill genomic DNA [50 ng DNA, 0.4 µM each primer, 150 µM each dNTP, 5 U *Taq* DNA polymerase, 30 cycles of 58°C annealing temperature for 30 seconds] analysis of the product on a 2% low melting point agarose gel revealed fragments approximately 1800, 1300, 800, 450 and 200 bp, as well as a smear of DNA fragments and primer dimers (Fig. 8). Since the actual site of amplification seemed to be somewhat ambiguous relative to the Spoonbill genome, a PCR amplification (same conditions) was performed on chicken DNA (*Gallus gallus*) to verify the primer specificity. The expected 205 bp (5A/5B) product was distinctly observed upon gel analysis of the product (Fig. 9). The primers SB-5A/5B still failed to give a specific homologous amplification product from Spoonbill genomic DNA, suggesting the sequences were inappropriate or that the amplification conditions needed to be further optimized.

As an additional control, primers 5A and T692-2 were used in an attempt to PCR amplify (same conditions as 5A/5B PCR) the expected 400 bp band from chicken DNA. This was also successful (Fig. 9) as were some other combinations of primers using chicken DNA templates (data not shown). However, when the same primer combinations were used with Spoonbill DNA, the expected product sizes relative to chicken DNA were not observed, whereas products ranging from 100 bp to 2,000 bp in length were obtained.

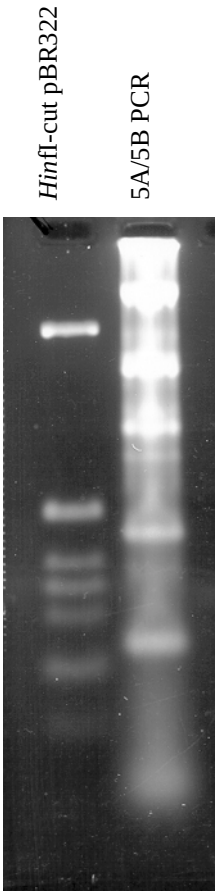

Figure 8. **Electrophoretic analysis of 5A/5B PCR amplified product from Spoonbill genomic DNA.** The PCR amplification of Spoonbill genomic DNA with primer set 5A/5B was gel electrophoresed in 2% LMP agarose at 40 V for 3 hours. The size standard is pBR322 digested with *Hin*fI.

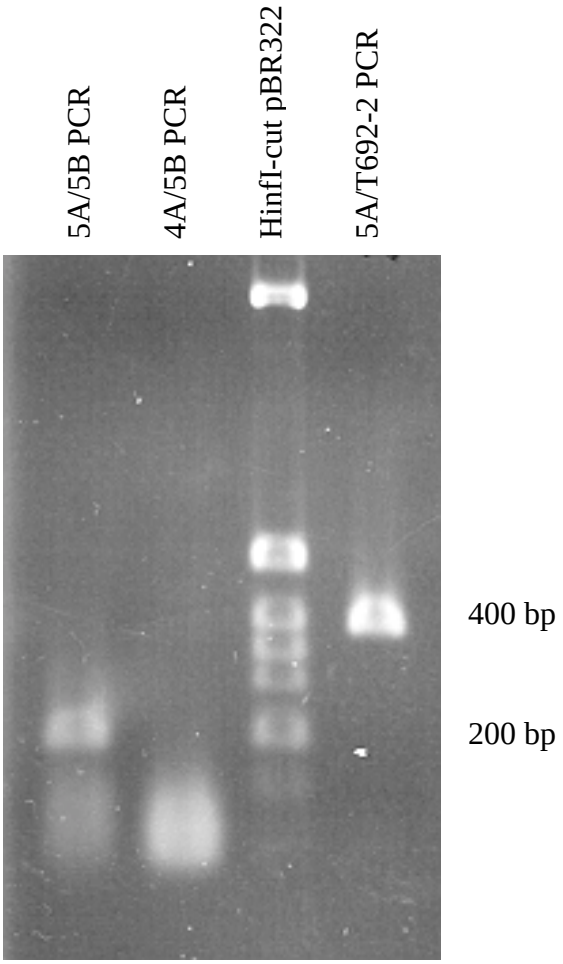

Figure 9. **Electrophoretic analysis of amplified chicken genomic DNA by MHC-locus primers.** The PCR amplification of chicken genomic DNA with 5A/5B primer set yielded a fragment of approximately 200 bp. PCR amplification with 5A/T692-2 primer set yielded a fragement of approximately 400 bp. Only primer dimers were evident from PCR amplification with the 4A/5B primer set. The DNA size standard is pBR322 digested with *Hin*fI.

Considering the limited success of directly amplifying small regions of interest from Spoonbill genomic DNA, the next logical step was to isolate a specific larger region of interest from a genomic library, which could then be screened for potential polymorphic regions. Larger regions or sections of DNA are usually acquired from a large insert phage or cosmid genomic library by use of an appropriate probe. I now possessed three potential probes for screening a Spoonbill genomic library (Fig. 10): two PCR products (approximately 200 and 400 bp) from the chicken MHC β1-chain and a 2.3 kbp chicken genomic clone (CCII-7-1).

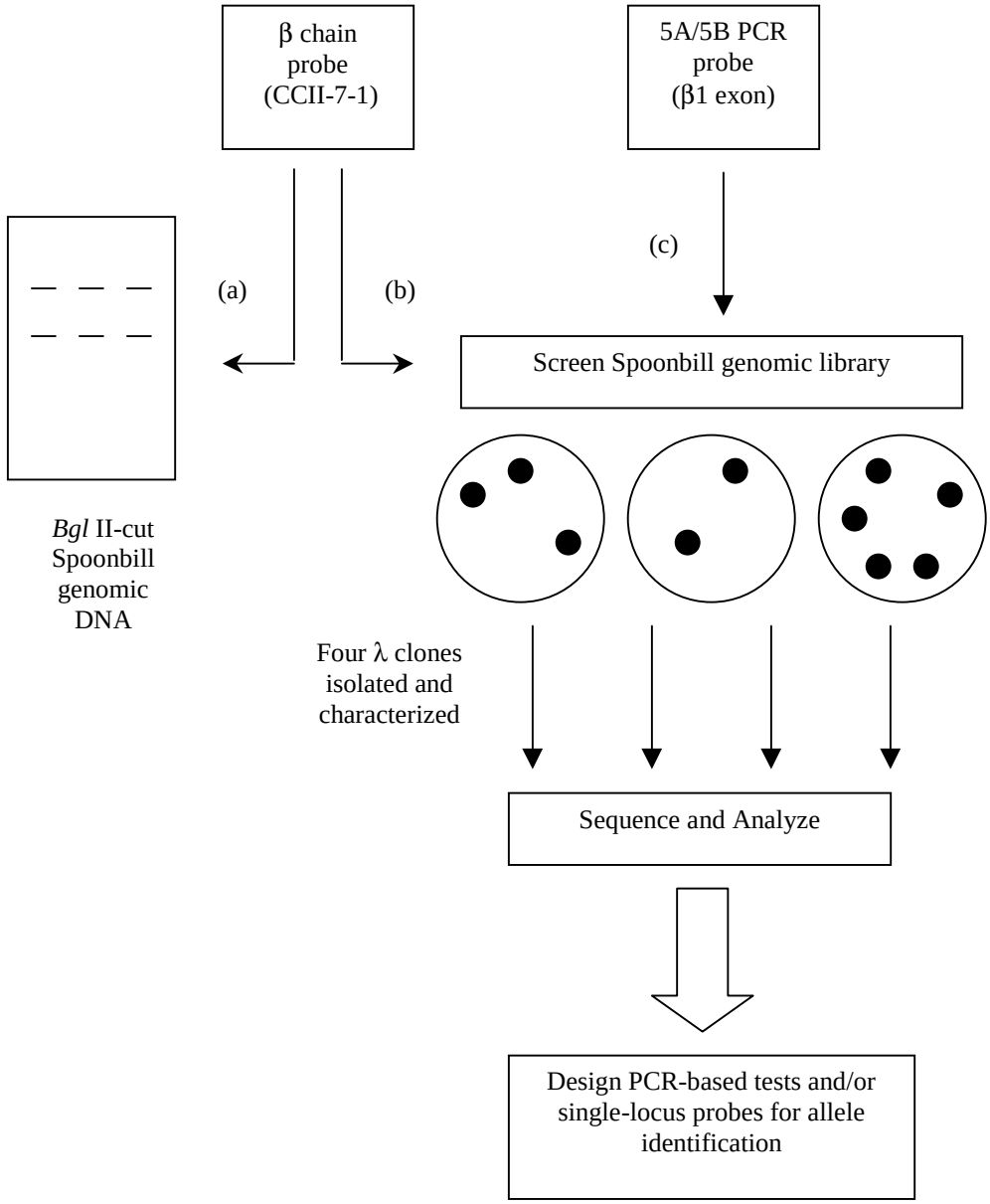
β chain probe (CCII-7-1)
5A/5B PCR probe (β1 exon)
(a)
(b)
(c)
Screen Spoonbill genomic library
Bgl II-cut Spoonbill genomic DNA
Four λ clones isolated and characterized
Sequence and Analyze
Design PCR-based tests and/or single-locus probes for allele identification

Figure 10. **Flowchart depicting the experimental options for using β-chain probes.** A 2.3 kbp genomic β chain probe (CCII-7-1) did not produce a polymorphic pattern (Fig. 7) when hybridized to a genomic blot of *Bgl*II-cut Spoonbill DNA (a). It was not used as a probe on the genomic library (b), but was available if needed. The 5A/5B (205 bp) PCR product from chicken DNA was used as a probe to isolate and characterize 4 clones from a genomic library (c).

Creation of a large insert Spoonbill genomic library

The isolation and characterization of a Spoonbill MHC gene, containing polymorphic regions, was still paramount. Since the PCR amplifications of chicken DNA did yield expected products, which potentially could be used as probes to isolate large DNA fragments from a genomic library, it was decided to construct a large insert (9-23 kbp) Spoonbill genomic library using recombinant lambda bacteriophage as the vector (Lambda DASH® II, Stratagene™).

The procedures outlined in Materials and Methods were followed closely with only minimal changes. In order to construct a Lambda DASH® II Spoonbill genomic library, genomic DNA was isolated from blood and subjected to the required procedures to prepare it for ligation with the lambda DNA. A pilot procedure involving the digestion of a small amount of Spoonbill genomic DNA with serially-diluted *Sau*3AI was performed (see Materials and Methods). The digestions of the genomic DNA samples gave the expected visible results as a gradient of partially digested DNA, which appeared as smears of continuously-sized fragments (Fig. 11). The DNA sample digested with the most enzyme (2 U/µg) contained a majority of DNA fragments in the size range of 3,000 bp and smaller. The DNA sample digested with the least amount of enzyme (1/128 U/µg) possessed the largest size fragments, most which were longer than 23 kbp. By comparing the fragment size ranges of the individual digestions, relative to the *Hin*dIII-cut lambda DNA markers, it was apparent that a true gradient of *Sau*3AI-digested genomic DNA samples had been produced. This was needed to be able to size-select the appropriate fragments from a preparative-scale partial digestion procedure.

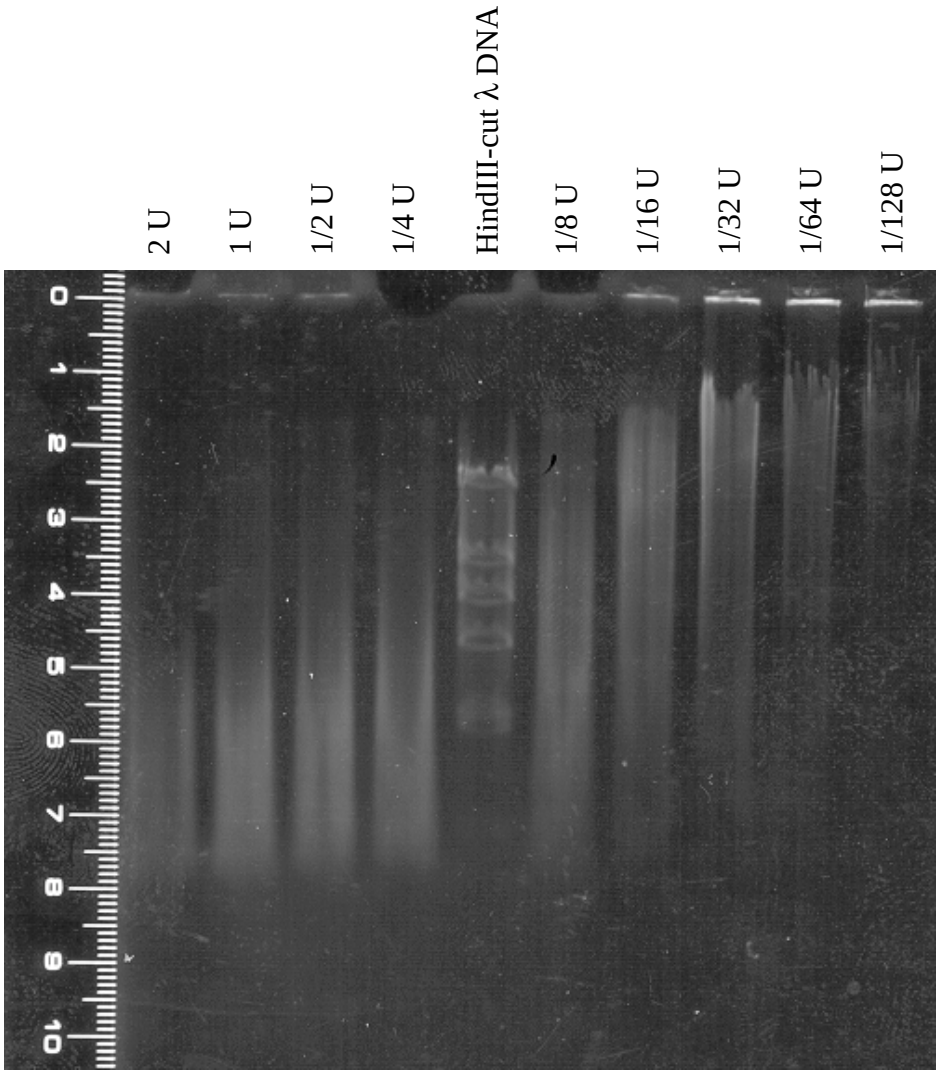

Figure 11. **Partial digestion of Spoonbill genomic DNA with *Sau*3AI.** The ruler on the left indicates centimeters of migration from the well. Each well contained 1 µg of cut Spoonbill genomic DNA with the units (U) of *Sau*3AI used in each digestion indicated above each well. The size standard is 1 µg lambda (λ) DNA digested with *Hin*dIII. The agarose gel consisted of a 1% base with a 0.3% top layer and was electrophoresed at 30 V for 14 hours at 4°C.

A preparative-scale partial digestion with *Sau*3AI was then performed on 80 µg of Spoonbill genomic DNA in a series of 8 microcentrifuge tubes (10 µg/tube). The first tube contained 20 U per 10 µg of *Sau*3AI and resulted in digestion of the majority of fragments to less than 2,000 bp (Fig. 12). The last tube contained 5/32 U per 10 µg of *Sau*3AI which resulted in a majority of fragments larger than 10,000 bp. A control microcentrifuge tube (Control) containing 10 µg genomic DNA and no enzyme was used to verify the DNA samples were digested with *Sau*3AI and not the result of random nuclease activity. The gel electrophoresis of Control DNA (Fig. 12) showed the presence of large fragments, all greater than 23 kbp, as expected for a nuclease-free sample.

The digested samples were individually cleaned and ethanol precipitated as previously described (see Materials and Methods). In an effort to exclude unwanted DNA from the final sample, that which would be too small or too large for proper insertion into the lambda arms (less than 9 kbp or greater than 23 kbp), only samples 5 U through 5/16 U, which contained fragments primarily in the size range of greater than 2 kbp and less than 23 kbp in length, were combined and loaded upon a prepared sucrose gradient. Centrifugation of the sucrose gradient was procedurally directed to occur at 26,000 rpm for 24 hours, for a total of 37,440,000 revolutions (Glover and Hames, 1995). However, due to multiple failures of the Beckman™ ultracentrifuge, only approximately 29,000,000 revolutions were completed. Even though the sample had only centrifuged for approximately 77% of the recommended time, it were processed in the hope that DNA fragment separation along the gradient had properly occurred. Portions of each fraction collected from the sucrose gradient were electrophoresed and the gel

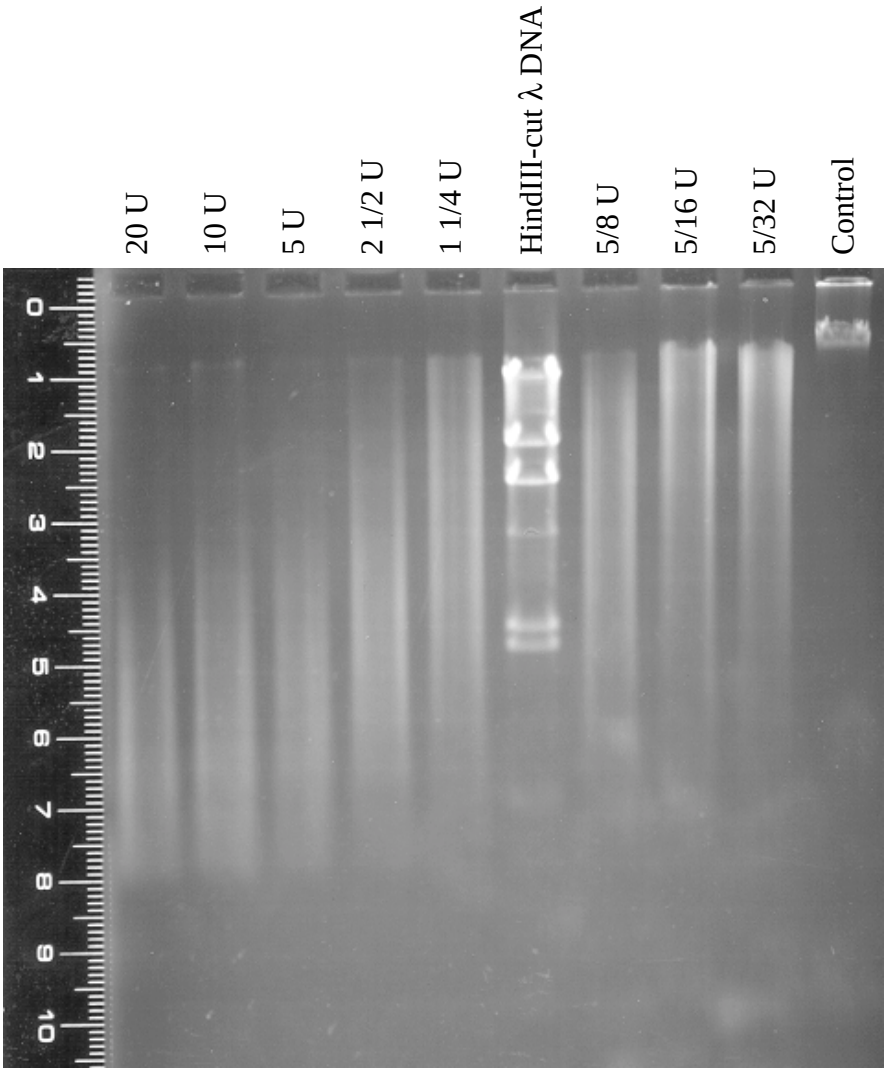

Figure 12. **Preparative-scale partial digestion of Spoonbill genomic DNA with *Sau*3AI.** The ruler on the left indicates centimeters of migration from the well. Each lane was loaded with 0.5 µg of Spoonbill genomic DNA digested with *Sau*3AI, with the units (U) used in each digestion indicated above each well. The last lane, "Control", contained uncut DNA (no *Sau*3AI) and the size standard is 1 µg lambda (λ) DNA digested with *Hin*dIII. The agarose gel consisted of a 1% base with a 0.5% top layer and was electrophoresed at 30 V for 15 hours at 4°C.

photographed as procedurally directed (data not shown). The fractions numbered 10 through 14 contained the desired size range of DNA fragments, approximately 9 to 23 kbp in length, and were pooled. The sucrose was removed from the pooled sample by affinity column chromatography (NENSORB™ 20, Dupont™) and the DNA was eluted in 100 µl TE, pH 8.0.

Agarose (0.7%) gel electrophoresis, 30 V for 10 hours, was performed with 5 µl of the eluted sample. A smear of DNA was evident from approximately 7 to 20 kbp (photograph not taken). In order to verify the quantity and purity of the DNA, 50 µl of undiluted DNA was analyzed, by use of a 100 µl total volume quartz cuvette, in a Beckman™ DU®-40 Spectrophotometer. The concentration was calculated to be 0.027 µg/µl, for a total recovered yield of 2.7 µg within the original 100 µl. The remaining sample was ethanol precipitated and resuspended in 4 µl ddH_2O. The ligation of 2 µl of this DNA to the pre-digested vector, and subsequent steps to create the packaged phage, was accomplished as directed in Materials and Methods. The library was titered and the results indicated a library containing 4.5×10^9 pfu/ml.

Screening the library for an MHC region

The choice of a probe and conditions for screening a genomic library is crucial, since one that is too short (e.g. a 30-mer oligonucleotide) may bind to multiple and unexpected regions of the genome, such as Pol III promoter "boxes" (Shariat, 1998). The 205 bp (5A/5B) chicken PCR product was first investigated as a potential probe by testing its specificity, with regard to binding to a limited number of loci in digestions of DNA from other species, by performing a zoo blot. The zoo blot consisted of one

human, one chicken and five Spoonbill DNA samples (2 μg each), which were completely digested with *Hae*III restriction endonuclease. The digested DNA samples were loaded onto a 1% gel and electrophoresed at 35 V for 16 hours. A DNA transfer (Southern, 1975) was performed to denature and affix the DNA to a membrane (Biodyne® A, GIBCO BRL®).

The 5A/5B (205 bp) probe was created by using Klenow fragment (New England Biolabs) to incorporate α-^{32}P-dCTP into the PCR product (100 ng) using the 5A/5B primer set to initiate synthesis. The probe was cleaned of excess nucleotides through spun chromatography with Sephadex® G-50. The probe was then hybridized to the membrane at 55°C for 16 hours. Two washes were performed at room temperature in 2X SSC, 0.1% SDS. The banding pattern observed on the autoradiograph showed encouraging results, with the probe binding to distinct regions within the Spoonbill genome (Fig. 13). The 5A/5B probe bound to the chicken genome in numerous places (8 distinct bands). The Spoonbill genome digests showed a single priming band at the same position in each sample plus three additional less intense bands at other positions. As might be expected, the human digest showed three very faint bands, probably due to the lack of a close relationship between birds and mammals. From these results it appeared that the 5A/5B probe was a good candidate for a β chain region probe for the Spoonbill genomic library.

The Lambda DASH® II Spoonbill genomic library was aliquoted to create 12 agar plates each containing 30,000 plaque forming units (pfu). This plaque density provided a confluent lysis of the host MRA(P2) cells. Two membrane lifts (A and B) were performed on each plate; SL1.0A and SL1.0B through SL12.0A and SL12.0B.

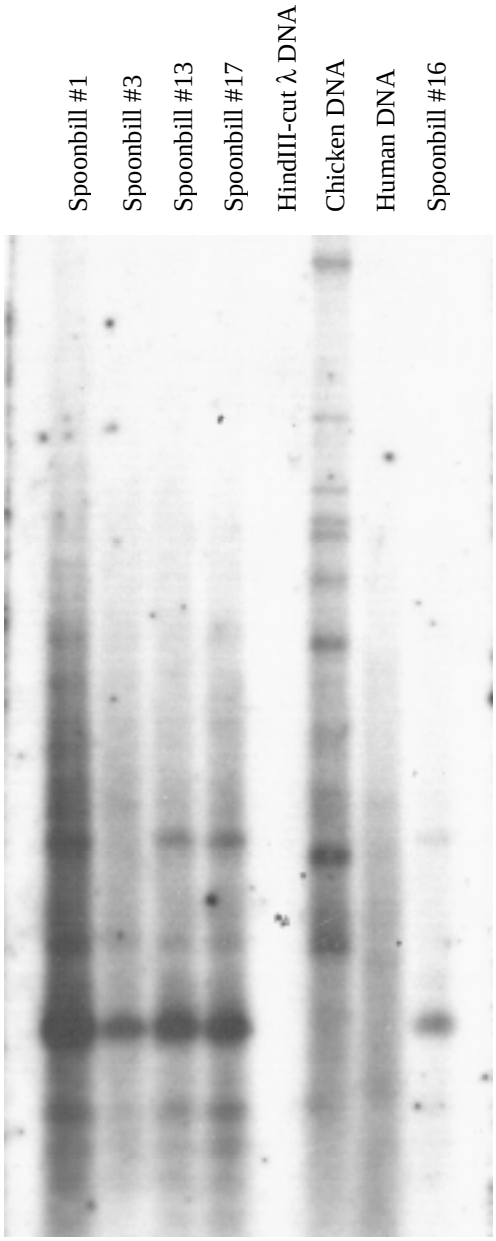

Figure 13. **Autoradiograph of a zoo blot**. Five Spoonbill DNA samples, 1 human and 1 chicken genomic DNA sample were digested with *Hae*III and gel electrophoresed on a 1% agarose gel at 35 V for 16 hours. The 5A/5B PCR amplified chicken DNA product (205 bp) was used as a probe on the Southern (1975) transfer of DNA from the gel.

Colony/Plaque Screen™ Hybridization Transfer Membranes (NEN™ Life Science Products) were used. The replica lifts were necessary to be able to properly identify positively hybridized clones, which would show hybridization of the probe to the same location on both the original and replica membranes. The lifts were hybridized at 55°C for 20 hours in batches of 4 to 8 membranes and 15 ml Hybridization Buffer per tube. The membranes were washed twice at room temperature for 15 minutes in 2X SSC, 0.1% SDS. Only 100-200 cpm were registered from each membrane by a Geiger counter. The membranes were individually covered and sealed with plastic wrap. The wrapped membranes were taped together onto a 14" x 17" inch piece of used X-ray film and allowed to expose a new film for 72 hours at –70°C with Cronex® Quanta III (Dupont) intensifying screens.

There was an average number of 2 positive "light-ups" per membrane. The range was from 0 to 6. Most of the positive light-ups were pulled for secondary screening by plating 20-500 pfu/plate. Tertiary and quarternary screenings were performed on plates with more than 500 pfu/plate to be sure positively hybridized and isolated clones were obtained. Four clones were selected for further characterization; SL10.4, SL9.4, SL15.3, SL16.3. These initial clones were each subjected to a large scale lambda clone preparation and purified by the use of cesium chloride equilibrium density gradient centrifugation. Analysis of each clone began with restriction mapping by digesting with *Eco*RI, *Pst*I, and *Xba*I and performing 1% agarose gel electrophoresis on the resulting products (Fig. 14).

Restriction mapping of the clones included the lambda arms of the DASH® II vector. This was not considered a problem since only the fragments of interest would be

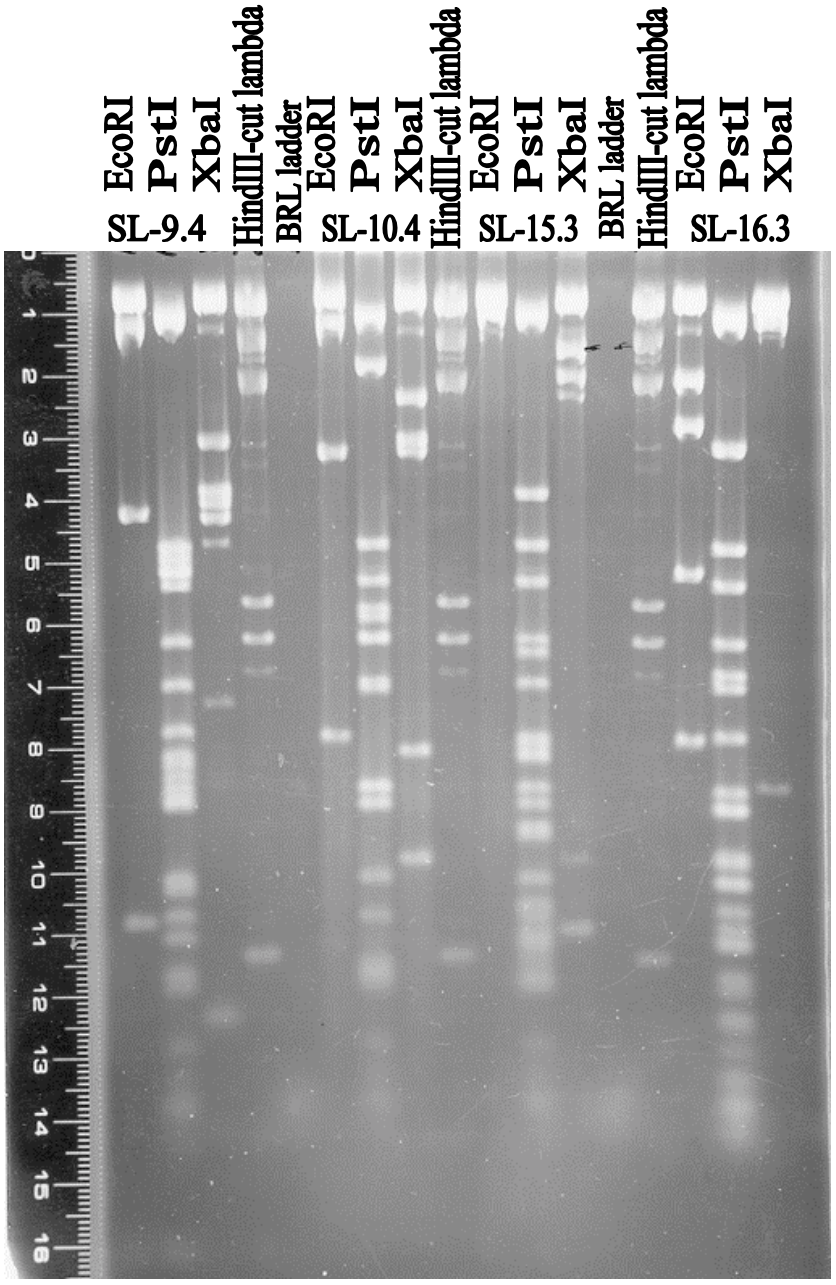
EcoRI
PstI
XbaI
HindIII-cut lambda
BRL ladder
EcoRI
PstI
XbaI
HindIII-cut lambda
EcoRI
PstI
XbaI
BRL ladder
HindIII-cut lambda
EcoRI
PstI
XbaI
SL-9.4
SL-10.4
SL-15.3
SL-16.3

Figure 14. **Electrophoretic analysis of restriction enzyme digested SL-clones.** Two micrograms from each of the four SL- clones (9.4, 10.4, 15.3 and 16.3) was digested with 1 of 3 different restriction endonucleases (*Eco*RI, *Pst*I, or *Xba*I) and gel electrophoresed at 30 V for 20 hours in a 1% agarose gel. The ruler on the left indicates centimeters of migration from the well. Two different DNA size reference standards were placed on the gel. *Hin*dIII-cut lambda is indicated. BRL® Ladder is commercially available (GIBCO BRL®) and is used as a hybridized ladder when needed.

selectively subcloned into a new vector. Identification of the fragments of interest was facilitated by the immediate Southern (1975) transfer of the fragments to a nylon membrane and hybridization with isotopically-labeled 5A/5B PCR product. An autoradiograph was produced with Fuji Medical X-Ray Film, RX (Fig. 15). The initial restriction mapping of the SL library clones was not sufficient to allow easy isolation and subcloning of all positively hybridized fragments.

A double digest of each phage clone was then performed, the products separated by gel electrophoresis and the gel stained and photographed (Fig. 16). Each clone was digested with *Eco*RI and another restriction endonuclease (*Bam*HI, *Hin*dIII, or *Sal*I). An autoradiograph was produced from hybridization of the 5A/5B probe with the Southern (1975) transfer containing the double digestion gel (Fig. 17). The banding patterns present on the autoradiograph were analyzed for the possibility of cloning targeted fragments into pUC18. It appeared that the double digestion products were not as useful as the single digestion fragments for the purpose of obtaining clones. However, the 1,600 bp bands created from the digestion of SL10.4 with *Eco*RI and each of the 3 enzymes, *Bam*HI, *Hin*dIII, or *Sal*I, were interesting since all three enzymes gave hybridizing fragments which appeared to be the same size. When the phage clone SL10.4 was digested with just *Eco*RI, it did not show a positively hybridized 1,600 bp band (Fig. 15). It was concluded that the three enzymes *Bam*HI, *Hin*dIII, and *Sal*I cut at the multiple cloning site of the lambda arm and *Eco*RI provided the other cleavage site, resulting in approximately equal length fragments.

Multiple sets of ligations were performed with digestions from each SL- clone in the attempt to subclone the proper regions of interest. Routinely, 24 rapid plasmid

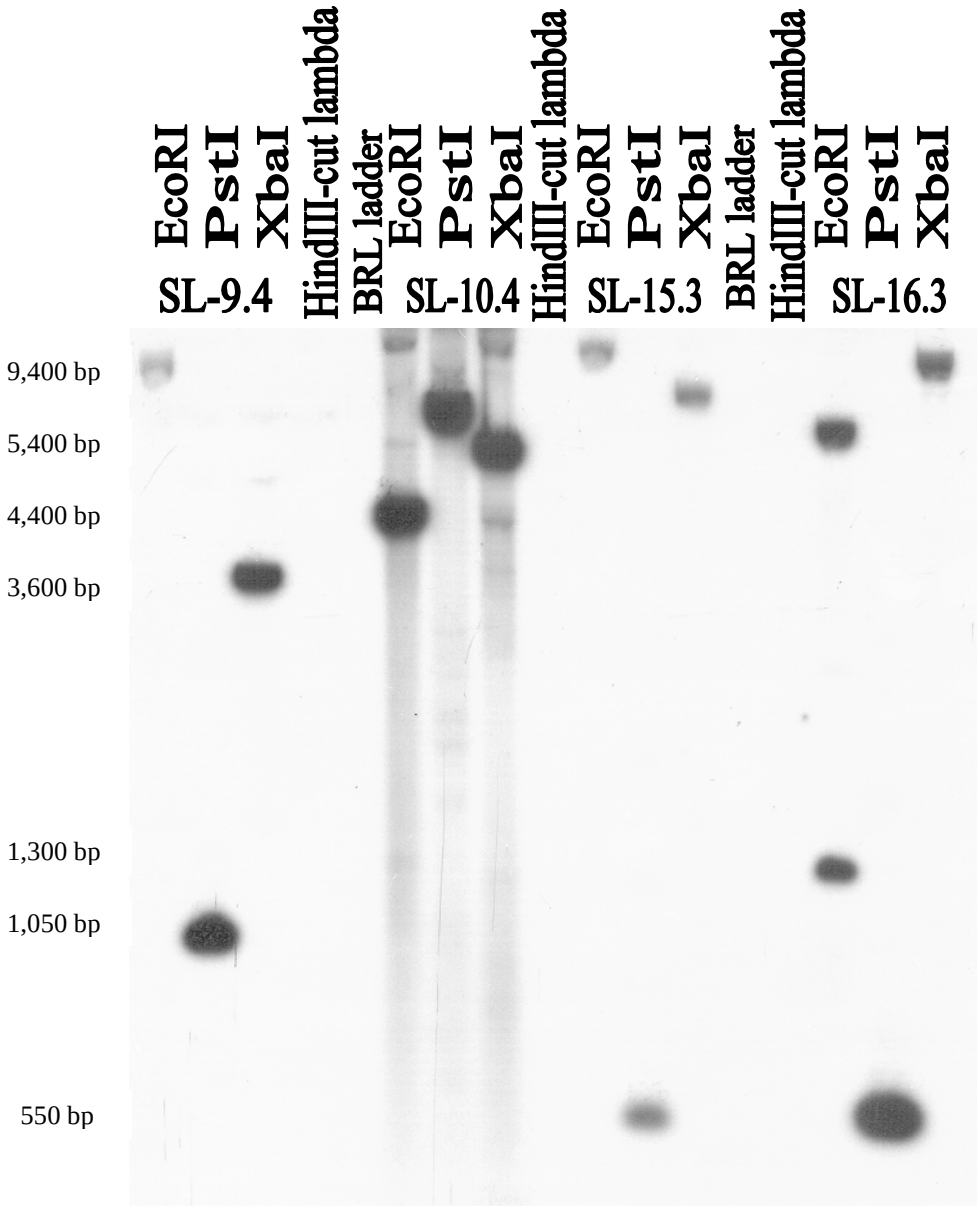

Figure 15. **Autoradiograph of the restriction-digested SL phage clones (Fig. 14) hybridized with isotopically-labeled 5A/5B PCR product**. Some positively hybridized fragments are labeled with their approximate size next to the autoradiograph.

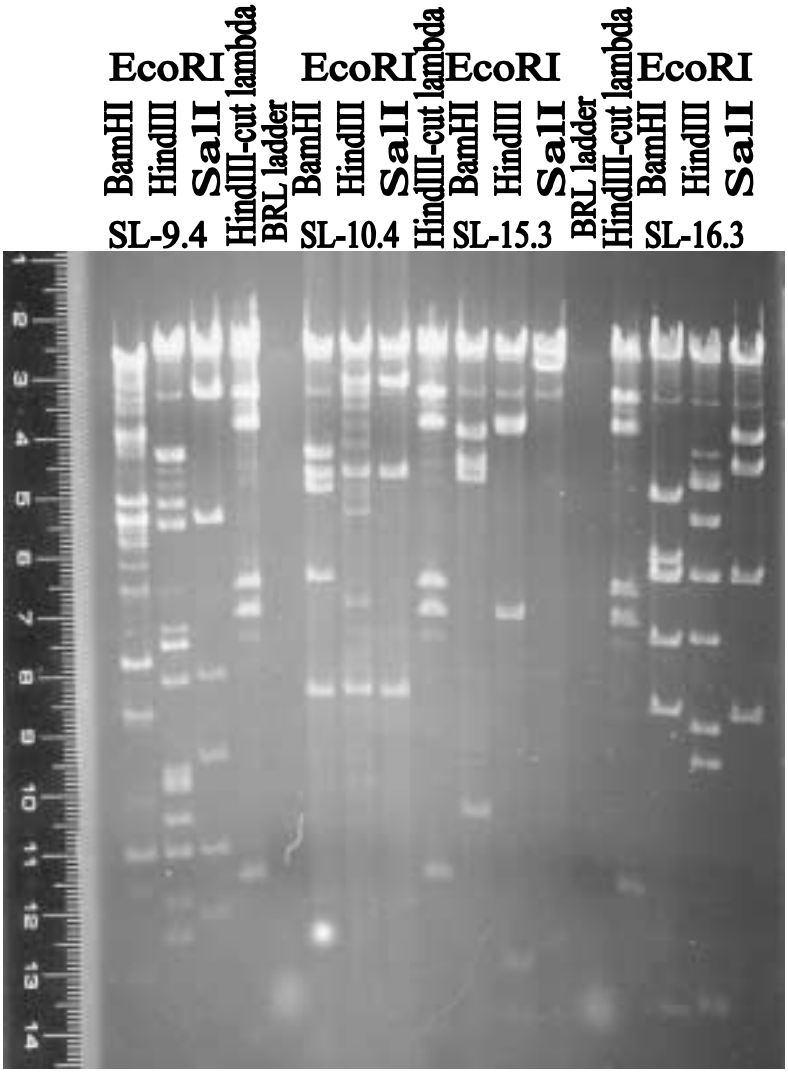

Figure 16. **Electrophoretic analysis of double-digested SL-clones.** Each of the four lambda SL- clones was double-digested with *Eco*RI and one of three other restriction endonucleases; *Bam*HI, *Hin*dIII, or *Sal*I. The samples were gel electrophoresed at 100 V for 5 hours in 1% agarose, then stained with EtBr and photographed. The ruler on the left indicates centimeters of migration from the well. Two different DNA size reference standards were placed on the gel. *Hin*dIII-cut lambda is indicated. BRL® Ladder is commercially available (GIBCO BRL®) and is used as a hybridized ladder when needed.

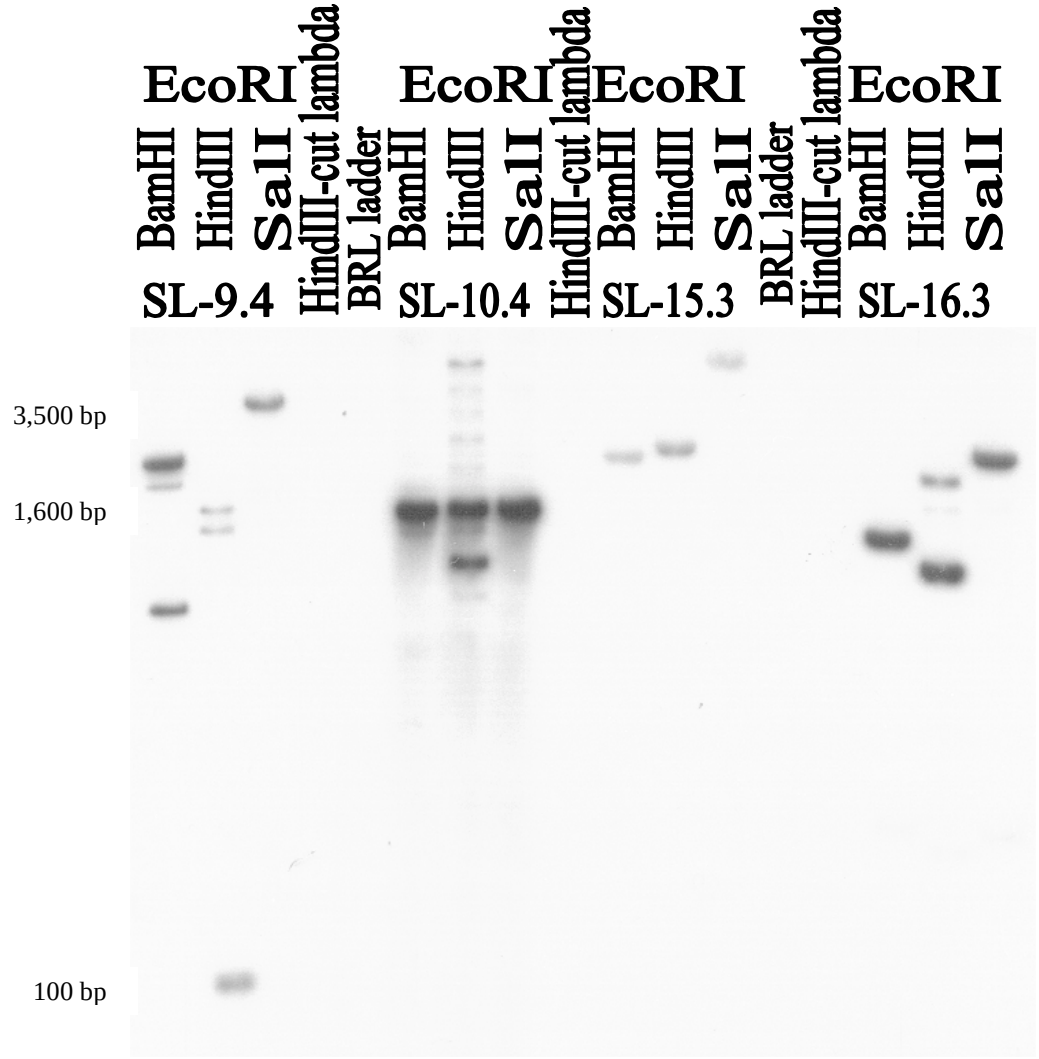

Figure 17. **Autoradiograph of double-digested lambda SL-clones (Fig. 16) hybridized with isotopically-labeled 5A/5B PCR product**. Representative positively hybridized fragments are labeled with their approximate size next to the autoradiograph.

isolations were performed on each set of ligation transformants, followed by restriction enzyme digestions, and agarose gel analyses to determine the sizes of DNA fragments cloned into the vector's multiple cloning site. Once a proper clone was identified by, for example, comparing its insert size to positively hybridized fragments (Fig. 15), a large scale plasmid isolation (see Materials and Methods) using cesium chloride was performed to provide high purity DNA so that future subclones could be created and DNA sequence analyses could be performed. Plasmids which contained inserts with relatively short (<1,500 bp) DNA sequences, were subjected to DNA sequence analysis using the M13/pUC forward (–40) and reverse (+24) sequencing primers.

An analysis of 24 samples, processed through the rapid plasmid isolation procedure and gel electrophoresed at 40 V for 5 hours, indicated only partial success in subcloning attempts (Fig. 18). Samples 1-12 represented attempts to subclone *Pst*I fragments from clone #30, a 1,300 bp *Eco*RI subclone of SL16.3 which contained an insert into the multiple cloning site of pUC18. Although this 1,300 bp clone contained a positively hybridizing fragment to the 5A/5B probe (the 1,300 bp *Eco*RI band present on the Fig. 15 autoradiograph), the *Pst*I digestion was an attempt to subclone the smaller 550 bp *Pst*I fragment which was visible on the SL16.3 *Pst*I lane of the autoradiograph. The *Pst*I 550 bp DNA insert was not subcloned.

Samples #21-#32 represented attempts to clone the 1,050 *Pst*I fragment from clone #3, an *Eco*RI subclone of SL9.4 which contained an 8,500 bp insert. Clone #22 (Fig. 18) appeared to contain the positively hybridized *Pst*I fragment of 1,050 bp in length, originally identified on the autoradiograph (Fig. 15). The DNA sequence of the 1,050 bp *Pst*I clone (#22) was determined to identify DNA homology with 5A/5B probe.

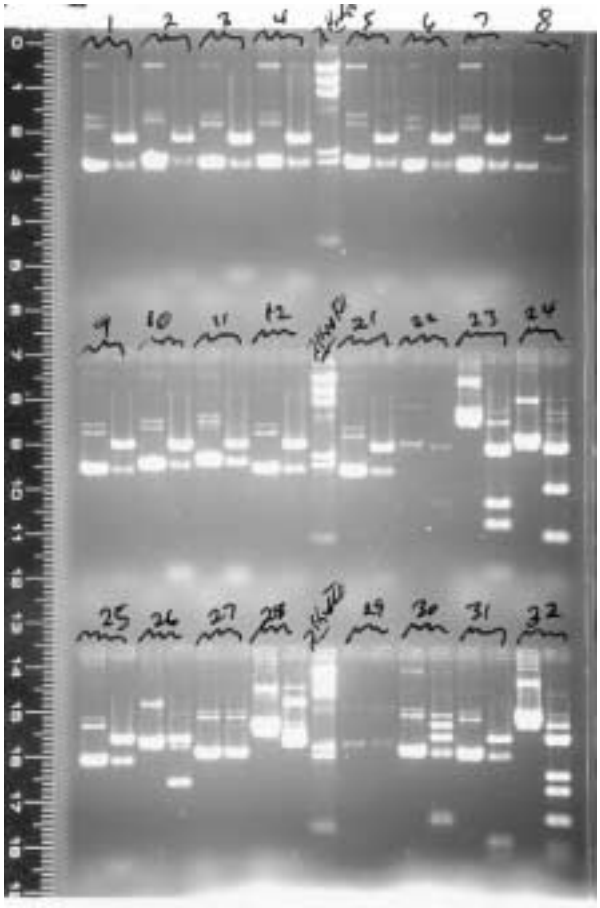

Figure 18. **Electrophoretic analysis of 24 rapid plasmid isolations from *Pst*I subclones of clone #3 (SL 9.4) and clone #30 (SL16.4).** Undigested (2 µg) and digested (2 µg) DNA samples from each plasmid isolation were loaded side-by-side on the agarose gel. The undigested DNA samples were loaded first (wells on the left), followed immediately by the *Pst*I digested samples. The 1% gel was electrophoresed at 40 V for 5 hours in 1X TAE. The ruler on the left indicates centimeters of migration from the well. The size standard, lambda digested with *Hin*dIII, is indicated by the notation λ*Hin*dIII.

Analysis of the DNA sequence determined that the SL9.4 subclone #22 actually carried a 1,128 bp insert. From the sequence homologies obtained from Basic Local Alignment Search Tool Program (BLAST®) (Altschul *et al*., 1997) in the GenBank database, it appears a highly homologous chicken MHC region was not obtained. Additionally, a simple homology comparison using the software DNASIS™ (Hitachi), showed the 5A/5B probe only matching 39%, with no particular region exhibiting a particularly high level of homology. However, nucleotides 39-154 of subclone #22 did show an 86% homology (BLAST® with GenBank) with nucleotides 15,754 to 15,954 (31,793 bp total submission) of the *Gallus gallus* T-cell receptor alpha chain gene, partial cds, and defender against death protein 1 (DAD1) gene, complete cds (accession #U83833). The 15,754 to 15,954 nucleotide region is associated with a CR1 (Chicken Repeat 1) element, not with any particular MHC gene, and is approximately 4,000 bp downstream from the chicken T-cell receptor alpha chain constant region (TCRAC) (Wang *et al*., 1997). CR1 elements are found throughout the chicken genome (~30,000 elements), reported to be from a class of non-long terminal repeat retrotransposons (Haas *et al*., 1997), and have been found in other avian genera as well as other vertebrate classes (Burch *et al*., 1993). Their relative abundance, and the use of the 5A/5B probe, may have allowed for the misidentification and cloning of putative Spoonbill β-chain regions.

The positively hybridized 1,300 bp fragment of SL16.3 was cloned. The DNA sequence of 712 bp was determined using universal forward and reverse primers. The DNA sequence obtained from the forward primer was 81% homologous (nucleotides 8-156) to a *Rattus norvegicus* microtubule-associated tubule 1A (GenBank accession

#M83196). There were no substantial matches in the database to the sequence obtained using the reverse primer. A subclone was created which contained a portion of the 550 bp *Pst*I fragment of SL16.3. The DNA sequence of 315 bp was determined, although no substantial regions homologous to the GenBank database were found. Interestingly, the SL15.3 clone carried a *Pst*I fragment of approximately 550 bp which hybridized well with the probe (Fig. 15). This fragment was cloned and the DNA sequence determined, which yielded a length of 609 bp. It has no major regions homologous to sequences in the GenBank database, and it only has a single segment of 35% homology with the 5A/5B probe. The 609 bp fragment is only randomly homologous (<40%) to the 315 bp fragment. Even though the 2 fragments appeared to be the same size (Fig. 15) and possibly the same cloned region, sequence comparisons indicate the possible similarity was a coincidence.

The positively hybridizing fragments of clone SL10.4 were 4,400 bp (*Eco*RI), 5,400 (*Xba*I), and 6,150 bp (*Pst*I). Because of their size, and the fact that the other cloned sequences from this effort did not yield regions highly homologous with the 5A/5B probe, attempts to subclone and determine their DNA sequences were not completed. In an effort to understand the failure to isolate the desired region in the Spoonbill genome, the 205 bp chicken PCR product was compared to GenBank, to which it matched exactly with the chicken MHC B-G antigen mRNA, as well as having limited matches with 30 other items. It is still unclear as to what portion of the probe bound to the fragments of interest. Based upon these results, it is obvious that higher stringency conditions (hybridization and washes at 60 to 65°C instead of 55°C) and/or more specific probes are necessary to isolate the desired MHC genes from a Spoonbill genomic library.

Additional primer sets for MHC PCR amplification

In order to continue the effort of trying to clone an MHC region, PCR primers Ex2a (38-mer) and Ex2b (29-mer) containing *Eco*RI sites (underlined) were specifically designed to amplify a 220 bp region of the polymorphic chicken B-LB (class II) genes (Zoorob *et al*., 1993).

Primer Ex2a: 5'-CCGAATTCGAGTGCCACTACCTGAAGGGCACCGAGCGG-3'

Primer Ex2b: 5'-CCGAATTCGCTCCTCTGCACCGTGAAGGA-3'

PCR reactions [10 ng DNA, 0.4 µM each primer, 150 µM each dNTP, 5 U *Taq* DNA polymerase, 30 cycles of 50°C annealing temperature for 45 seconds] using these primers with chicken genomic DNA did yield the expected product of 220 bp (low yield), as well as two other bands (low yield) at approximately 800 and 1,200 bp (Fig. 19). However, a distinct product of approximately 1,600 bp was obtained from some of the Spoonbill DNA samples (#1 and #3), with one individual (#1) yielding a 1,200 bp amplicon in addition to the 1,600 bp product (Fig. 19). The PCR products were ethanol precipitated and digested with one of three restriction endonucleases (*Eco*RI, *Alu*I or *Pst*I) in an attempt to clone the PCR products into appropriately cut pUC18. Some clones were created, however, only portions of the 1,200 bp amplicon resulted in short regions of homology (BLAST® search of GenBank, Altschul *et al*., 1997) with *Mus musculis* or *Gallus gallus* MHC regions (data not shown). Since the expected PCR amplification product of 220 bp was not amplified for Spoonbill DNA, while 1,200 and 1,600 bp products were produced, it was assumed the presence of introns within the Spoonbill genomic DNA resulted in the larger than expected products.

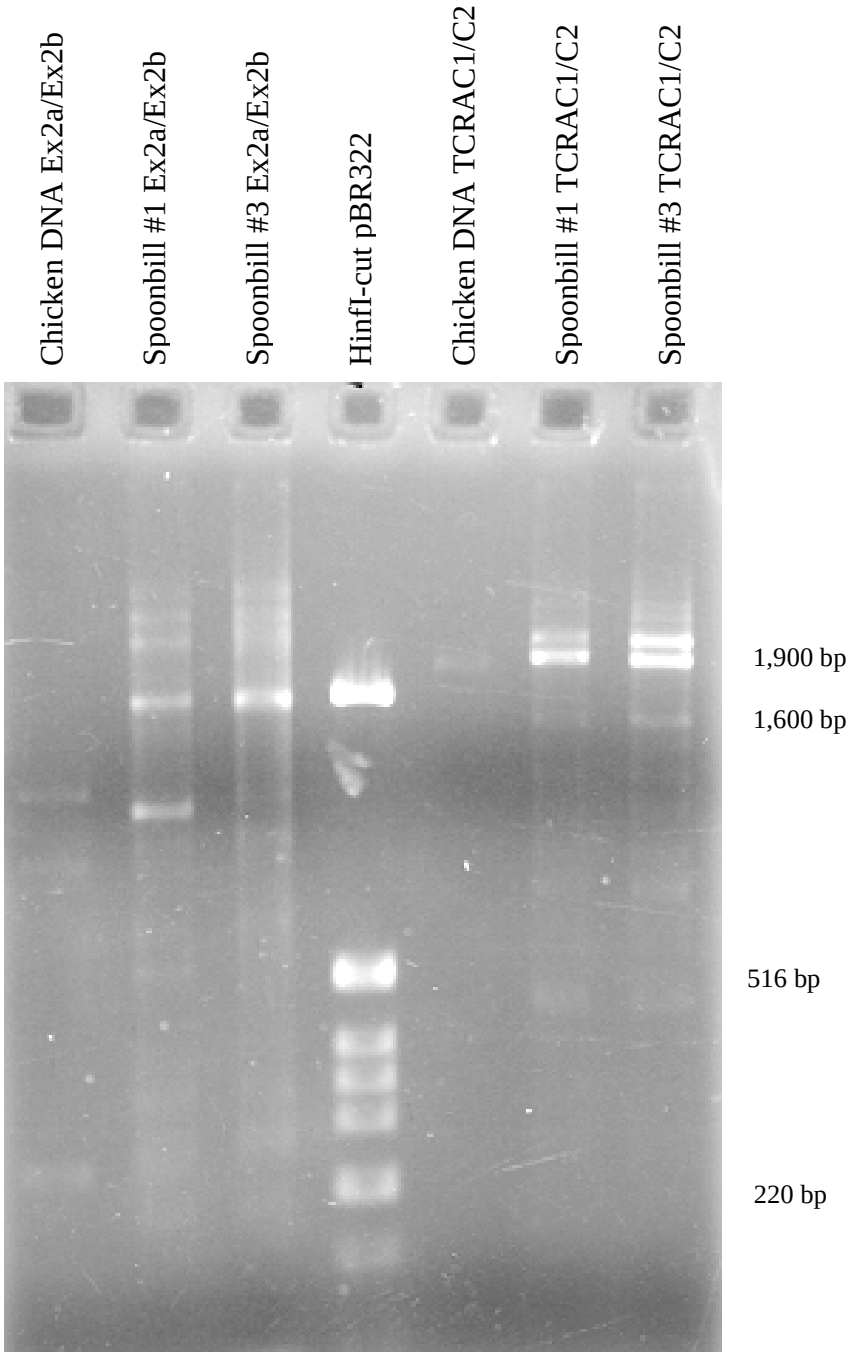

Figure 19. **Electrophoretic analysis of Ex2a/Ex2b and TCRAC1/C2 PCR-amplified products.** The amplification products were electrophoresed on a 2% agarose gel at 80 V for 1.5 hours. The size standard is the plasmid pBR322 digested with *Hin*fI.

The PCR primers TCRAC1 (21-mer) and TCRAC2 (21-mer) were then designed to amplify a 320 bp portion of the extracellular (C ex) exon segment of the chicken 1.7 kbp candidate T-cell receptor alpha (TCRα) cDNA (Gobel *et al*., 1994).

Primer TCRAC1: 5'-ACTCCATCTCCTTCAGTCTAC-3'

Primer TCRAC2: 5'-CATGTTCAGGTTCTCATCTGT-3'

This region of the T-cell receptor was expected to be highly conserved between chickens and Spoonbills. The PCR product (amplicon) obtained from PCR amplification [10 ng DNA, 0.4 µM each primer, 150 µM each dNTP, 5 U *Taq* DNA polymerase, 30 cycles of 50°C annealing temperature for 45 seconds] of chicken DNA was approximately 1,900 bp. Two products were obtained from initial amplifications of Spoonbill DNA, approximately 1,900 and 1,950 bp (Fig. 19). Modified PCR amplification conditions [only 0.5 U *Taq* DNA polymerase and the annealing temperature was raised to 60°C for 45 seconds and 35 cycles] intensified the 1,900 bp product and did not amplify the 1,950 bp product. Attempts to clone the PCR product directly into blunt-cut (*Sma*I) pUC18 were not successful. The two primers were then redesigned with the incorporation of *Eco*RI sites at the 5'-ends.

Primer TCRAC1B: 5'-CCGAATTCACTCCATCTCCTTCAGTCTAC-3'

Primer TCRAC2B: 5'-CCGATTCATGTTCAGGTTCTCATCTGT-3'

PCR amplification, using the higher stringency condition listed above, with Spoonbill DNA yielded the expected amplified product (1,900 bp) containing *Eco*RI restriction endonuclease sites at the 5'-ends. The product was cleaned of excess nucleotides, primers and buffer by use of the Quantum Prep® PCR Kleen Spin Columns (Bio-Rad Laboratories). Portions of each cleaned PCR product (about 1 µg) were then

independently digested with three restriction endonucleases (*Eco*RI, *Pst*I, or *Hae*III) to determine which fragments would clone well and yield useful sequences. The digested fragments were electrophoresed on a 2% agarose gel at 80 V for 1.5 hours (Fig. 20). The fragments were ligated with appropriately-digested pUC18 and then transformed into DH5α™. Rapid plasmid preparations (12), followed by restriction enzyme digestions and 1% agarose gel analyses, were performed to identify the DNA inserts, relative to the digested PCR fragments. The DNA sequences of the subclones were determined by the Sanger and Coulson (1978) dideoxy chain termination method. This method of subcloning multiple overlapping fragments, typically referred to as contigs (contiguous fragments), and determination of the DNA sequences from each, is colloquially known as "shotgun" cloning and sequencing. The software DNASIS™ (Hitachi) facilitated alignment of homologous contig regions to create the full size PCR amplification product of 1,942 bp. This T-cell receptor α 1 clone (TCRα1) was submitted to GenBank and has the accession #AF452238.

Comparison of the complete Spoonbill product (1,942 bp) to the GenBank database (BLAST® search, Altschul *et al*., 1997) identified multiple regions of homology (Table II), ranging from 83% (nucleotides 455-776) to 97% (1116-1153) relative to: *Gallus gallus* T-cell receptor alpha chain gene, partial codons, and defender against death protein 1 (DAD1) gene, complete codons (accession #U83833). Various Spoonbill samples (#1, #3, #7, #10, #11, and #18) were amplified with the TCRAC1B/TCRAC2B primer set and each sample yielded the approximately 1,900 bp band. There was no obvious difference in size (polymorphism) among the Spoonbill PCR products. In order to detect single nucleotide polymorphisms (SNPs) between samples, each individual

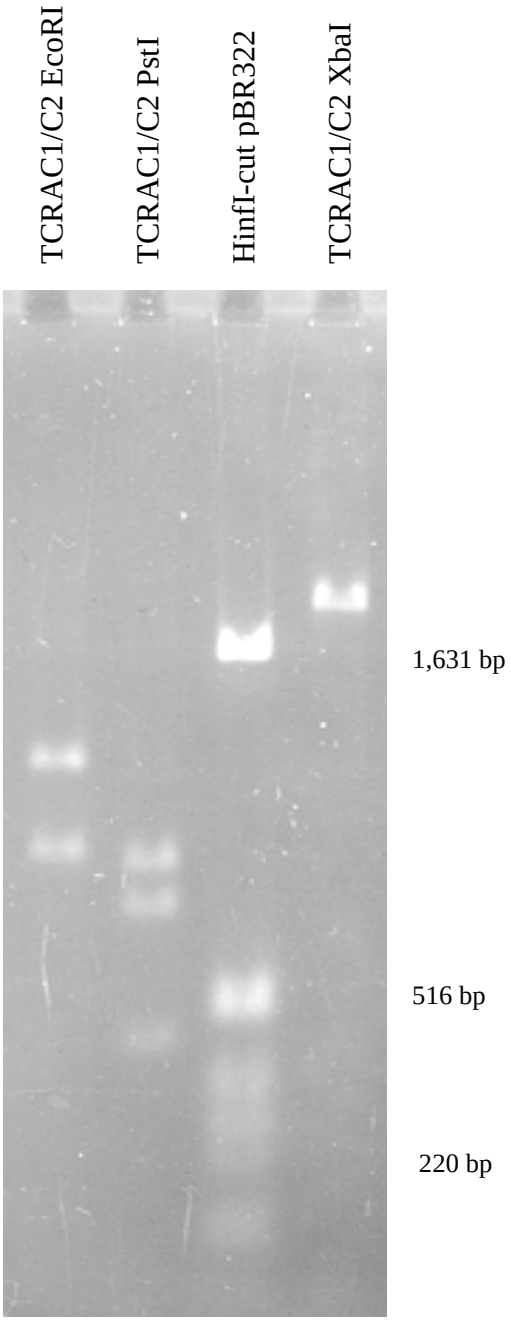

Figure 20. **Electrophoretic analysis of TCRAC1/C2 amplified products from Spoonbill #7 genomic DNA.** The TCRAC1/C2 PCR product was independently digested by *Eco*RI, *Pst*I, and *Xba*I and electrophoresed in a 2% gel at 80 V for 1.5 hours. The size standard is plasmid pBR322 digested with *Hin*fI.

Spoonbill amplicon would require DNA sequence determination, followed by comparisons to detect single nucleotide differences. Further attempts to find polymorphism between individuals by this approach were suspended in order to focus on sex determination by PCR amplification and development of enrichment techniques to isolate STR loci from genomic DNA.

Table II. **BLAST® homologies of Spoonbill TCRα1 (accession #AF452238) with the chicken T-cell receptor alpha chain gene, partial codons, and defender against death protein 1 (DAD1) gene, complete codons (accession #U83833).**

Nucleotide positions	Homology
1-71	90%
105-190	94%
285-350	89%
455-776	83%
1116-1153	97%
1313-1353	92%
1358-1401	93%
1878-1935	89%

Sex determination in Roseate Spoonbills

Determination of the sex of an animal has importance dating back to the first domesticated animals. By choosing the correct sex, a breeding pair could be established to produce more offspring and prevent the need to round up or buy others. This practice continues today by ranchers, zoo personnel, wildlife managers, as well as any other individual who is responsible for maintaining animals either in large herds or in small captive groups. The capability to properly manage an avian population, either in the wild or in a zoo, may be complicated by the lack of obvious sexual dimorphism. The sex type of some birds is visually apparent in some species such as the cardinal, with the male hosting the red plumage and females the brown. In other species, sexual dimorphic characteristics, such as when males are larger than females, are evident only when both sexes are in proximity, as with the Carribean (American) flamingo, *Phenocopterus ruber ruber* (Chris Brown, personal communication). For captive populations, especially where reproduction is high, the gender ratio should be determined as quickly as possible so that the unwanted bird/chicks can be culled, thereby reducing the cost of feeding and housing them.

One zoological technique, which was routinely used in zoos (and is still used periodically today) to determine the sex of birds in which sexual dimorphic characteristics were not obvious, utilized the invasive technique of surgery (endoscopy) under general anesthetic to inspect for the presence of ovaries/testes. The "surgical sexing" procedure has limitations which include (1) the possibility of the bird's death, (2) the expenses to cover the surgical team, and (3) the margin of error (incorrect sexing) which is variable depending upon the team performing the surgery (Laurie McGivern,

personnel communication). Even karyotyping birds has a limited value in determination of sex since the sex chromosomes, ZZ in males and ZW in females, are not dimorphic in many species. Given the expense and time needed to anesthetize and perform surgery on one bird, it was fortuitous when sex determination became available by DNA tests. Companies such as Avian Biotech now offer a DNA-based sex typing service, for a fee of $20 per sample, for practically any species of animal.

The discovery of the first avian W chromosome gene was achieved by Griffiths and Tiwari (1993). Their experiments showed that the chromo-helicase-DNA (CHD)-binding gene in mice had its chromosomal location on both sex chromosomes in birds. Furthermore, differences between the intron sizes on the W and Z chromosomes could be found (Fridolfsson and Ellegren, 1999; Griffiths *et al*., 1998). A PCR-based test was designed by Griffiths *et al*. (1998) which took advantage of the size differences between the introns of the CHD-binding genes on the W and Z chromosomes. Primers P2 and P8 (Table III) were designed to PCR amplify the same region from both chromosomes to create two products of different length, for example 300 versus 350 bp. Use of these primers in other avian species resulted in chromosome specific products from their W and Z chromosomes, except in the Ratitae birds (ostriches, emus, cassowaries and others). Although the chromosome-specific PCR amplified products obtained from some bird species were not always easily distinguishable from each other, since the size differences were very small, it was hypothesized that these oligonucleotides may be used as universal primers to sex type most species of birds, except the ratites (Griffiths *et al*., 1998).

Table III. **Oligonucleotide primer sets designed to universally differentiate avian sex chromosomes by PCR amplification.**

P2: 5'-TCTGCATCGCTAAATCCTTT-3'

P8: 5'-CTCCCAAGGATGAGRAAYTG-3' R = A/G Y = T/C

P2/P8 primer set by Griffiths *et al*., 1998

2550F: 5'-GTTACTGATTCGTCTACGAGA-3'

2718R: 5'-ATTGAAATGATCCAGTGCTTG-3'

2550F/2718R primer set by Fridolfsson and Ellegren, 1999

The P2/P8 primer set was used in attempts to sex type Roseate Spoonbills and Caribbean (American) Flamingos (*Phoenicopterus ruber ruber*). The PCR amplification of genomic DNA extracted from whole blood from each species resulted in products of approximately 400 bp, as seen after 2% agarose gel electrophoresis (80V, 1.5 hours). These products appeared to be informative (flamingo results not shown) only after visualization of them on a non-denaturing polyacrylamide gel (Fig. 21). A single band of approximately 390 bp resulted from the amplification of the Z chromosomes, hence male birds. The samples with 2 bands (approximately 390 and 396 bp) resulted from genomes containing the Z chromosome as well as the W chromosome, hence female. Separation of 2 bands from 1 band was dependent upon the gel electrophoresis conditions. A problem with some aspect of the testing procedure became evident when sample #33 was

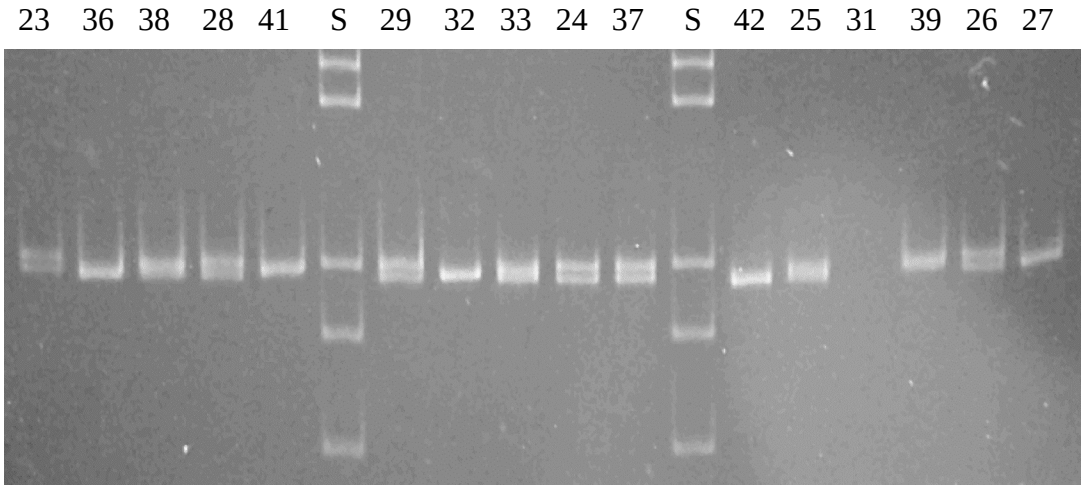

Figure 21. **Electrophoretic analysis of Dallas Zoo Spoonbill genomic DNA samples amplified with the P2/P8 primer set.** The amplified products were electrophoresed on a 7.5% (37.5:1) gel for 12 hours at 100 V. The listed Spoonbill sample numbers can be correlated with zoo identification numbers using Table V. The size standard "S" is pBR322 digested with *Hin*fI. The sizes of the ladder fragments are (from top to bottom): 516, 506, 396, 344, and 298 bp.

reported to be female, by a surgical sexing procedure, whereas analysis of the DNA results evident from the gel electrophoresis showed only one band, and was therefore a male. Since the true sex of the individual and the usefulness of the "universal" P2/P8 primers were questionable, each of these problems was addressed independently.

To verify the reported sex of Spoonbill #33, the Dallas and Sedgwick County Zoos were contacted. Each zoo had on record the surgical sexing of the bird as female. A copy of the report indicates the procedure was performed on February 15th, 1996 at the Spoonbill's age of 7 months and 10 days (report generously provided by Jamie Kirk, Sedgwick County Zoo, Kansas). Since Spoonbill #33 was reported to be female, gel electrophoresis conditions were slightly modified (16 hours, 100 V) in the attempt to visualize the any presence of a faint second band (Fig. 22). Analysis of the 7 PCR amplified products on the gel revealed the appearance of 4 male samples. Only sample #38 was supposed to be male. Three (#31, #33, and #34) of the 6 zoo-identified females appeared to be males when PCR amplified with the P2/P8 primer set.

One technique to differentiate between the Z and W chromosome PCR products, obtained using the P2/P8 primer set, is to digest the PCR products with restriction endonucleases (Griffiths *et al*., 1998), followed by agarose gel electrophoresis and visualization of the male/female DNA patterns. In an attempt to keep DNA-based sex testing as simple and efficient as possible, this method was not employed. Fortuitously, for this study, Fridolfsson and Ellegren (1999) had designed multiple primers from other regions of the CHD gene in order to be used as universal primers in sex typing birds. One set of primers, designated 2550F and 2718R (Table III), was obtained and used in an

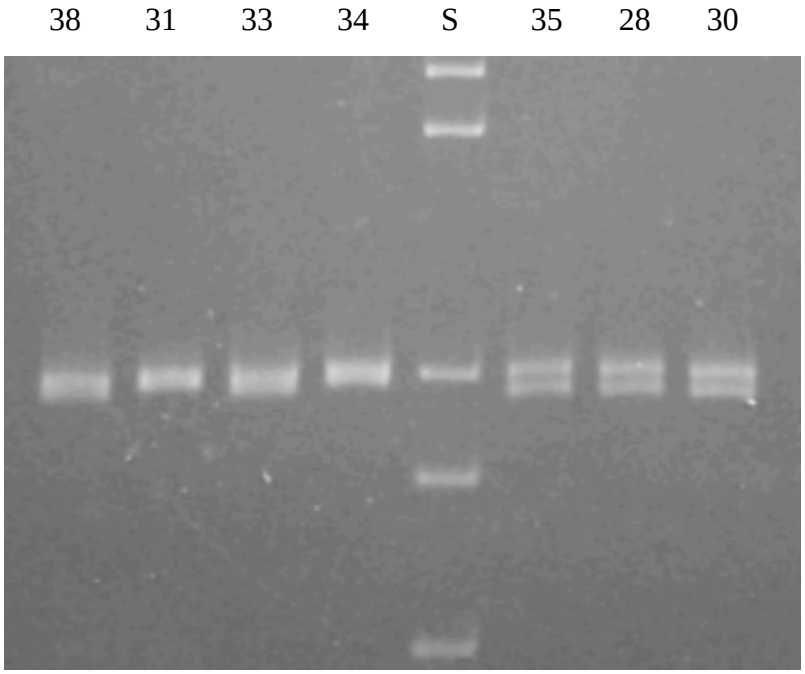

Figure 22. **Electrophoretic analysis using modified conditions of Dallas Spoonbill genomic DNA samples amplified with the P2/P8 primer set.** The amplified products were electrophoresed on a 7.5% (37.5:1) gel for 16 hours at 100 V. The listed Spoonbill sample numbers can be correlated with zoo identification numbers using Table V. The size standard "S" is pBR322 digested with *Hin*fI. The sizes of the ladder fragments are (from top to bottom): 516, 506, 396, 344, and 298 bp.

attempt to PCR amplify DNA from Spoonbills, as well as from Caribbean (American) Flamingos (to evaluate their cross species utility). The PCR parameters used to amplify DNA with the P2/P8 primer set were modified to work with these new primers (Fridolfsson and Ellegren, 1999). The primer annealing temperature was decreased by 1°C every cycle from 60°C to 50°C, continuing with 30 cycles at the 50°C annealing temperature, followed by 5 minutes at 72°C, and finally a constant hold at 4°C. This "step-down" PCR procedure amplified a product of approximately 450 bp from female Spoonbills (data not shown). Amplification of the female Flamingo samples produced 2 products, an expected Z chromosome product of about 450 bp and another product (from the W chromosome) at approximately 700 bp. Additional Spoonbill samples were ampified, which consistently produced the 450 bp product for females and a 700 bp product for males. The absence of an amplified Z chromosome product (700 bp) from the DNA of female Spoonbills was explained by the preferential amplification of the 450 bp product (Fridolfsson and Ellegren, 1999).

The results, male or female designation, from using the P2/P8, 2550F/2718R and/or Aajμ4 (Table XII) primer sets in PCR-based sex determinations are recorded in Tables IV, V, and VI. The 2550F/2718R PCR amplification product of Spoonbill #33 resulted in a band at about 700 bp, indicating a male Spoonbill. This result was not considered definitive, since the chromosome CHD-specific region could be mutated at the primer site by just a few nucleotides. Such a mutation may prevent proper primer binding on the W chromosome, resulting in the lack of a 450 bp band being produced by PCR amplification, and preferential amplification of the 700 bp band. The mutated primer binding site may also explain the results of the P2/P8 primer set (only one band

Table IV. **Sex Type of Fort Worth Zoo Roseate Spoonbills**			
Sex type determined via PCR with the P2/P8 and Aajμ4 primer sets			
*Sample #	Fort Worth Zoo	Sex Type	Sex Type
	bird ID	in zoo records	determined
			via PCR
1	950616	UNKNOWN	female
2	950605	UNKNOWN	male
3	950607	UNKNOWN	male
4	950617	UNKNOWN	male
5	950604	UNKNOWN	female
6	950606	UNKNOWN	male
7	950620	UNKNOWN	female
8	950619	UNKNOWN	female
9	950610	UNKNOWN	male
10	O579	male	male
11	O235	male	male
12	O584	female	female
13	930501	male	male
14	940574	male	male
15	O240	female	female
16	O485	female	female
17	930576	female	female
18	970610	UNKNOWN	male
19	O1577	female	female
20	940639	male	male
21	O527	male	male
22	930633	female	female
*Sample # assigned arbitrarily			

Table V. **Sex Type of Dallas Zoo Roseate Spoonbills**

Sex type determined via PCR with P2/P8, 2550F/2718R and Aajμ4 primer sets

Note: Spoonbill #33 was incorrectly surgically sexed

*Sample #	Dallas Zoo bird ID	Sedgwick Co. Zoo bird ID	Sex Type in zoo records	Sex Type determined via PCR
23	823308		Female	female
24	823309		Female	female
25	823311		Male	male
26	833689		Female	female
27	844071		Male	male
28	854407		Female	female
29	854411		Female	female
30	854414		Female	female
31	928065	5442	Female	female
32	94C135	6756	Male	male
33	95C136	7222	FEMALE	MALE
34	95C137	7224	Female	female
35	96C139	7639	Female	female
36	OOD401		UNKNOWN	male
37	OOD402		UNKNOWN	female
38	OOD404		UNKNOWN	male
39	OOD406		UNKNOWN	female
40	OOD407		UNKNOWN	male
41	OOD408		UNKNOWN	male
42	OOD437		UNKNOWN	male

*Sample numbers arbitrarily assigned

Note: #33 bill length is 17.0 cm (this is another independent test to verify sex type)

Table VI. **Sex Type of Sedgwick County Zoo Roseate Spoonbills**				
Sex type determined via PCR with 2550F/2718R and Aajμ4 primer sets				
*Sample #	Sedgwick Co. Zoo bird ID	Dallas Zoo bird ID	Sex Type in zoo records (bill length)	Sex Type determined via PCR
43	2668	864681	female (15.0 cm)	female
44	3454		male (17.2 cm)	male
45	3471	886242	female (15.7 cm)	female
46	3473	886232	male (17.5 cm)	male
47	5422	928062	female	female
48	6745		male	male
49	7637	96C138	female	female
50	9619		UNKNOWN	male
51	9628		UNKNOWN	female
*Sample numbers arbitrarily assigned				

visible), if in fact another band from the W chromosome product should be present, where only one band was observed.

In an effort to utilize another independent test to verify the sex of Spoonbill #33, a request was submitted to the Dallas Zoo's Roseate Spoonbill studbook keeper, Laurie McGivern, to measure the bill of the bird. Jamie Kirk at Sedgwick County Zoo had informed me of the technique by which sex may be determined by measuring the bill, where the length and width of the bill is measured in centimeters, and the length may indicate the sex of a bird. Laurie's review of some specimen reports, where the length of the bill and the sex of a bird are known, allowed her to hypothesize that Spoonbills with a 16.2 cm bill or shorter are female, whereas males possess bills in the range of 16.5-18.2

cm (Laurie McGivern, personal communication). The bill length of Spoonbill #33 was 17.0 cm, which is comfortably within the range expected from a male Spoonbill.

Since Spoonbill #33 was confirmed to be a male bird by the three methods described above, independent of the male results obtained from the P2/P8 PCR amplification, the misleading DNA-typing results (from P2/P8 PCR amplification) of Spoonbills #31 and #34 were then analyzed. The PCR primer set P2/P8 was used for the amplification and electrophoresis of these Spoonbill samples (Fig. 22) and the results are interpretable as male for both (single band), even though the Dallas zoo has them listed as female. The zoo records do not indicate why Spoonbill #31 was known to be a female. Spoonbill #34 was surgically sexed as female on February 15th, 1996, the same day as Spoonbill #33.

The PCR amplifications of Spoonbills #31 and #34 with the 2550F/2718R primer set showed the products of 450 bp bands, interpretable as female for both (photo not taken). The results of the PCR amplifications with the sex-linked microsatellite primer set, Aajμ4 (Table XII), also indicated the sex of Spoonbills #31 and #34 as female. Therefore, two independent tests confirmed the female sex status on file at the Dallas Zoo for both individual Spoonbills. From these results, it appeared the P2/P8 primer set was not reliable as a sex indicator for two Roseate Spoonbills DNA samples. A restriction endonuclease study (a digestion of the PCR products with *Hae* III or *Mae* II) may have proved to differentiate the products from a P2/P8 PCR amplification (Griffiths *et al*., 1998). However, two other DNA-based tests had been shown to be useful for sex determination in Roseate Spoonbills. Therefore, optimization of an unneeded and more tedious DNA test, such a restriction endonuclease digestion of the P2/P8 PCR products,

was not a practical use of time and resources, especially for a technique which may never work properly.

The possibility of a direct genetic link between the 2 female samples (#31 and #34) was investigated, especially since the parents of one or both may carry the genetic anomaly (if the mutation was not spontaneous). Nine blood samples, and their corresponding Specimen Reports, were acquired from the Sedgwick County Zoo, specifically from some of the Spoonbills related to the Dallas Spoonbills. Spoonbill #31 was reportedly a half-sibling of #34 and possibly a full-sibling of #35. Spoonbill #31 was reportedly the offspring of either dam #43 or #45. Analyses of the DNA profiles created from the STR testing of 51 Spoonbills (Tables XII and XXI) allowed the determination that #45 was not the dam of #31, and either #44 or #43 was not a parent. However, analyses of the DNA profiles of #43, #44 and #45, relative to #35, indicated that none of them could be a parent of #35, which left it as having no known relationship to #31.

Spoonbills #31 and #34 reportedly had the same sire, #44. An analysis of the STR profiles supported the reported data (Table XXI). A simple explanation for the reason why 2 female Spoonbills, from the same sire but maybe different dams, carried the same mutant allele was that the sire passed on the allele. Therefore, an analysis of the lineages of the female samples was performed in order to eliminate them as the genetic donors. This question was asked: Was the mutant allele inherited from the dams? The dam of Spoonbill #34 was #2670 (Sedgwick Co. ID; bird died and tissue was not available) and the dam of #31 may be #43. Both dams were born at the Dallas Zoo, #2670 in August 1985 and #43 in May 1986, which means they could have been full or half siblings (parents of either were not recorded). This conjecture helped explain the

lack of genetic diversity within the Dallas Zoo (Linn, 1993), as well as provided a possible inheritance pattern for the mutant allele within 2 different Spoonbills with lineages through the same Zoo, Dallas.

Since the parents of #44 (collected from the wild) and either dam (#43 and #2670) were not recorded, determination of the origin (founder) of the mutated CHD region was going to be difficult, if not impossible. Whenever it was possible, the inheritance pattern(s) of the mutant allele was extrapolated. A visual analysis of the P2/P8 amplification products of Spoonbill genomic DNA (e.g. samples #23, female, and #36, male, Fig. 21) indicated that the lower band (about 390 bp) was amplified from the Z chromosomes and the upper band (about 390 bp) was amplified from the W chromosome. The amplification products of Spoonbills #31 and #34 were single upper bands (Figs. 22 and 23), even though they were female and should have produced 2 bands. The Z chromosome CHD locus could have mutated in order to not produce the expected lower band. If this hypothesis was correct, the mutant allele was a Z-linked allele and could only be passed on to female offspring by male sires. This hypothesis was supported by the evidence that Spoonbill #44 sired the 2 females (#31 and #34), both which contained the mutant allele.

DNA from Spoonbill #43 (possible dam of #31) was amplified to produce the expected 2 bands from a female (Fig. 23). Since #43 did not appear to carry the mutant profile (presence of only a single band), the inheritance of the mutant allele by Spoonbill #31 must have originated from the sire: Spoonbill #44. This is because a W-linked allele would have to be inherited by the female offspring, otherwise it would not become a

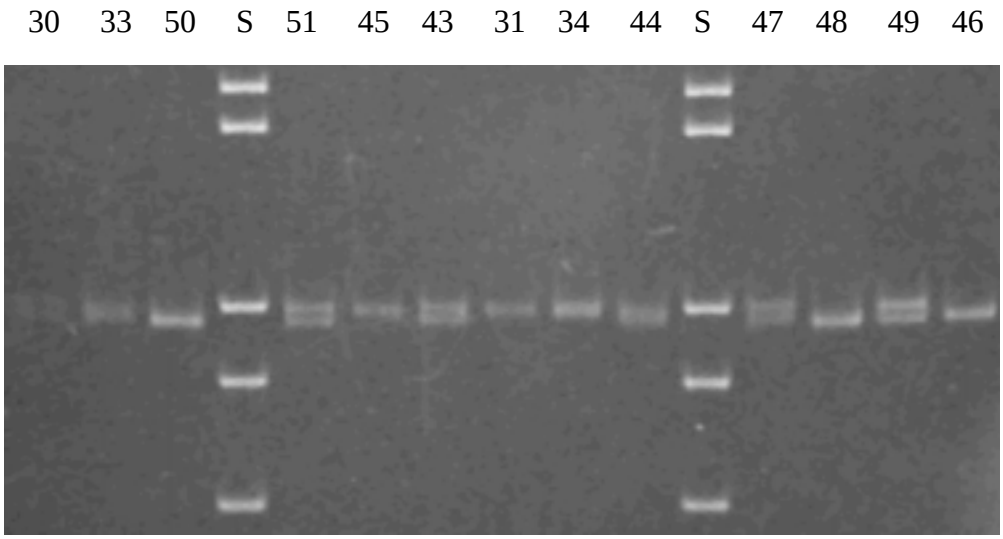

Figure 23. **Electrophoretic analysis of Dallas and Sedgwick County Zoo Spoonbill genomic DNA samples amplified with the P2/P8 primer set.** The PCR amplified products were electrophoresed on a 7.5% (37.5:1) gel for 17 hours at 100 V. The listed Spoonbill sample numbers can be correlated with zoo identification numbers using Tables V and VI. The size standard "S" is pBR322 digested with *Hin*fI. Fragment sizes from top to bottom (in bp): 516, 506, 396, 344, and 298.

female. Since dam #2670 was not available, DNA samples were obtained from 2 half-siblings of Spoonbill #31: #47 and #49 (both female). These offspring were a result of #2670 mating with Spoonbill #46. If the mutant allele was inherited from #2670, on the W chromosome, then #47 and #49 would carry the mutant allele. These DNA samples were amplified and electrophoresed (Fig. 23). Two bands were produced from both #47 and #49. Therefore, #2670 could not have had the mutant allele, and Spoonbill #31 could not have inherited it from #2670.

These results were possible under the hypothesis of a Z-linked allele. Additionally, since the presence of the mutant profile was not found in the dams of Spoonbills #31 and #34, the mutant allele could not have been W-linked. With the support of the above evidence, it was accepted that the mutant allele was Z-linked. Since male birds have two Z chromosomes, the possibility of sire #44 passing on a mutated chromosome to some offspring (#31, #34 and possibly #33) and a normal chromosome to another, was entirely possible (if it was heterozygous for the alleles). Since Spoonbill #44 was the mutant allele carrier, then PCR amplification with P2/P8 may reveal the presence of 2 bands (normal and mutant) or 1 band (homozygous mutant). The amplification product of #44 was not a single narrow band (normal alleles), but it also was not two bands or even a single distinct band (Fig 23). The amplification product of Spoonbill #33 was not a single Z chromosome band. In fact, it appeared to be very similar to the band produced from the amplification of #44 (Fig. 23).

The amplification product of Spoonbill #45 (female) was an unexpected single band, indicative of a male, and possibly contained the similar or same mutant allele as #31 and #34. Spoonbills #45 (female) and #48 (male) were the documented parents of

#51. The Z-linked hypothesis for the mutant allele could be tested by asking this question: Could a female offspring (#51) inherit the mutant phenotype of the P2/P8 amplification? The amplification of Spoonbill #51 DNA produced the 2 bands expected for a female (Fig. 23). It appeared that the Z-linked hypothesis was still supported by the evidence.

The lineage of Spoonbill #45 was investigated since it produced the same or similar mutant profile as #31 and #34. Spoonbills #30 (dam) and #854412 (sire) were the documented parents of offspring #45 (born June 13, 1988). However, #30 could not have been the dam of #45 since DNA profiles (via PCR of STR loci) provided the exclusionary evidence (Table XXI). Spoonbill #854412 was born in the wild (West Nueces Bay, Texas), approximately early May 1985. Since the dam was not properly identified, the sire (#854412) was considered to be the carrier of the mutant allele. The documented sire (#44) of Spoonbills #31 and #34 were also born in the wild (Louisiana coast), approximately late April 1985. All of the above evidence supports the hypothesis that the mutant alleles were inherited from the male Spoonbills (#854412 and #44). Spoonbill #45 inherited the mutant allele from #854412. Spoonbills #31 and #34 inherited the mutant allele from #44.

An estimation of the relative frequency of the mutant allele within the wild population would be pure conjecture. The evolutionary significance of not being able to differentiate the Spoonbill PCR products resulting from the amplification with the P2/P8 primer set may never be fully understood. It is most likely, because of the lack of evolutionary pressure on non-coding regions to remain the same, that the mutation occurred within the intron of the Z chromosome and therefore, no deleterious effects will

be realized. However, by analyzing the genetic lineages of the 3 female Spoonbills (#31, #34, and #45), the possible carrier #33, and the sires which passed on the mutant allele (#44 and #854412), Spoonbill studbook keepers can look for increases or decreases in genetic fitness (offspring production/survival rates), relative to other Spoonbills.

Evolutionary study of the CHD sequences

Some avian phylogenetic relationships have been developed based on the DNA sequences determined from CHD-W and CHD-Z PCR amplifications with the P2/P8 primer set (Garcia-Moreno and Mindell, 2000). Since neither Spoonbills, nor Flamingos were included in the study by Garcia-Moreno and Mindell, the homologous DNA sequences of a male Spoonbill and a male flamingo were determined and made available within the GenBank database. Sequences have been deposited in GenBank under accession numbers AF440750 (Fig. 24, Spoonbill) and AF440751 (Fig. 25, Flamingo).

```
10         20         30         40         50         60
GTGTGTTTAG TTTGTTTCTT GGGGGTTGTT GTTGGGTTTT GGTTTTTGCA CTGGTTTTTG

        70         80         90        100        110        120
TTTTTGTTTT TTTTCCCTTT TCTGAACACA CGTTTTTGAC AGGCTAGGTA GAACTTTACT

       130        140        150        160        170        180
TTTGTTCGTA GCTTTGAACT ATTTATTCTC AAATTCCAGA TCAGCTTTAA TGGAAGTGAA

       190        200        210        220        230        240
GGGAGGCGCA GTAGGAGCAG AAGATACTCT GGATCTGATA GTGACTCTAT CTCAGAAAGA

       250        260        270        280        290        300
AAAACGGCCA AAAAAACGTG GAAGACCACG CACTATTCCT GAGAAAATAT TAAAGGATTT

       310        320        330        340        350        360
AG........ .......... .......... .......... .......... ..........
```

Figure 24. **302 bp nucleotide sequence from P2/P8 amplified Roseate Spoonbill (*Ajaia ajaja*) genomic DNA.** This sequence was submitted to GenBank and assigned the accession number AF440750.

```
10         20         30         40         50         60
GCTGTTAGTT TAGTCTGGTT GTTGGGTGTT GTTGTTGGGT TTTGGTTTTT GGATTGTTGC

        70         80         90        100        110        120
TTATTGCCTT TCCTGAACAC ATGTTTTTGA CAGGCTATAG GTAAAACTTT ACTTATGTGT

       130        140        150        160        170        180
GTTAATCGTG TAGCTTTAAA CTACTTCTGA AATTCCAGAT CAGCTTTAAT GGAAGTGAAG

       190        200        210        220        230        240
GGAGGCGCAG TAGGAGCAGA AGATACTCTG GATCTGATAG TGACTCCATC TCAGAAAGAA

       250        260        270        280        290        300
AACGACCAAA AAAACGTGGA AGACCACGAA CTATTCCTCG AGAGAATATT AAAGGATTTA

       310        320        330        340        350        360
GCGA...... .......... .......... .......... .......... ..........
```

Figure 25. **304 bp nucleotide sequence from P2/P8 amplified Caribbean (American) Flamingo (*Phoenicopterus ruber ruber*) genomic DNA.** This sequence was submitted to GenBank and assigned the accession number AF440751.

STR isolations from the Roseate Spoonbill genome

STR loci are often highly polymorphic because they typically do not exist in exons and are therefore not constrained by evolutionary pressure to stay the same over time. Their polymorphic nature is evident as the number of repeats vary at a particular locus when compared amongst individuals (Li *et al*., 1997). These size differences can be quickly determined by PCR amplification and gel analysis of the products, or by the detection of fluorescent-labeled products (*GenePrint*® Fluorescent STR Systems, Promega).

The STR enrichment technique (Fig. 1) using paramagnetic particles was used repeatedly to isolate microsatellite regions from the Spoonbill genome. Each subsequent attempt was modified slightly in the effort to increase the specificity of STR capture. Significant changes, relative to the procedure outlined in Materials and Methods, are described below. Once the potential STR-containing clones were "captured", they were transferred into *E.coli* DH5α™ by following the transformation protocol. Each transformant (white colony) had the potential of carrying a clone harboring an STR region. The DNA sequence of each clone was determined in order to conclusively identify the presence of an STR region.

The first STR isolation attempt deviated substantially from the final outlined procedure in Materials and Methods. The differences begin with the oligonucleotide adapter construction. An oligonucleotide labeled "EcoLinker" (5'-CCGGAATTCCGG-3') was annealed to itself and then ligated to 5 µg (Spoonbill #17) of restriction endonuclease digested genomic DNA. Three enzymes were used (*Alu*I, *Hae*III, and *Rsa*I) to create blunt-ended genomic fragments. After the self-annealed EcoLinker was

ligated to the blunt-ended fragments, oligonucleotide STR-1 was used in the capture technique. Ultimately, the captured fragments were digested with *Eco*RI to create fragments with "sticky" ends, which should have ligated efficiently into the *Eco*RI-cut vector (pUC18). These clones were then transformed into DH5α™ and spread-plated onto 82-mm LB-Ampicillin, 50 µg/ml, agar plates. This first attempt to isolate STR loci yielded approximately 100 small white colonies. DNA was isolated from 24 colonies by following the rapid plasmid isolation procedure. Each of the 24 DNA samples was digested with *Eco*RI for 2 hours at 37°C. The samples were then electrophoresed in a 1% agarose gel at 80 V for 2.5 hours (Fig. 26). Only 6 samples (identified as: 2, 3, 5, 6, 10 and 11) appeared to have a distinct insert into the multiple cloning site (MCS) of pUC18. The DNA sequence of each sample was determined using the Sequenase™ version 2.0 (United States Biochemical) DNA sequencing kit with the forward (-40) and reverse (M13R) sequencing primers. The DNA inserts ranged in size from 125 bp (#3) to 340 bp (#10). However, none of the samples carried an STR.

The second attempt to capture STR loci used 5 µg of Spoonbill #18 genomic DNA digested with *Sau*3AI. An oligonucleotide adapter was created by annealing the S61 olgonucleotide to S62. The captured fragments were ligated to the vector, pUC18, at the *Bam*HI site. Transformation of the DNA clones resulted in 57 blue colonies and 16 white colonies. Each of the 16 white colony transformants was processed via the rapid plasmid preparation procedure to prepare the DNA samples for screening and sequencing analyses. The screening procedure which tested for the presence of a DNA insert was modified because the protocol had been altered when *Sau*3AI-cut genomic DNA was ligated into a *Bam*HI-cut pUC18. Screening of the white colonies for possible inserts

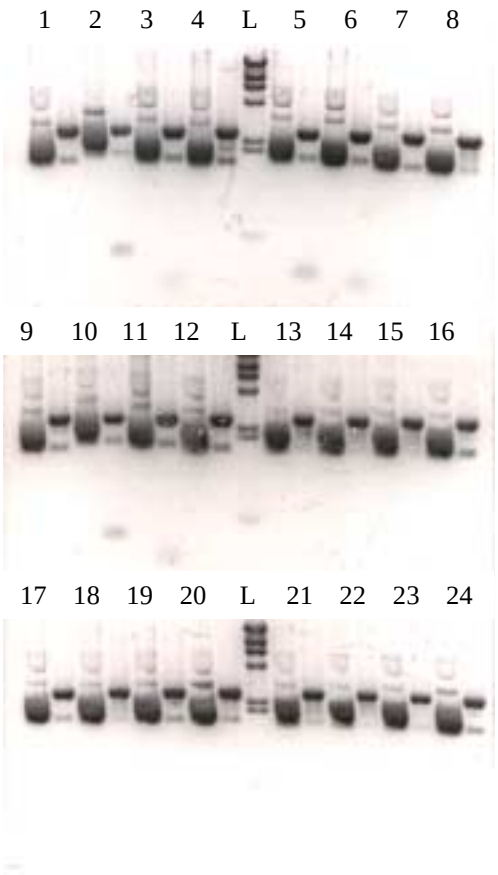

Figure 26. **Electrophoretic analysis of 24 *Eco*RI digested and undigested plasmid DNA samples from the 1st STR capture attempt.** Plasmid DNA samples 1-12 were from the 6/2/99 transformation and 13-24 were from the 6/3/99 transformation. The "L" designates the size standard: lambda DNA digested with *Hin*dIII.

into the MCS could not be done by digestion with *Sau*3AI or *Bam*HI, because neither restriction endonuclease would cut out the entire DNA insert from the MCS. The alternative technique of colony PCR was employed to screen the DNA samples for the presence of an inserted DNA fragment, as well as the presence of any STR loci.

Each transformant colony, or a very small portion of bacterial cells taken from a streak on a master plate, was assayed by the colony PCR procedure (see Materials and Methods) by using three primers: the forward (-40), reverse (M13R), and STR-1 [biotin-GAATAT$(GGAT)_4$]. These three primers were supposed to PCR amplify the entire DNA insert region, as well as produce any product between an STR locus and the forward or reverse primers. The products obtained from PCR amplification were analyzed by loading them on a 1% agarose gel and electrophoresing them at 60 V for 3 hours (Fig. 27). Visual inspection of the photographed gel showed the presence of DNA inserts, of the approximate size of 300 to 600 bp, in most of the transformant samples. None of the samples appeared as two bands, which would have been indicative of the presence of an STR within the clone. In the interest of trying to understand which type of clones were captured, the DNA sequences from each of the 16 samples were determined by using the universal forward (-40) and reverse (M13R) primers, relative to pUC18.

The transformant clone 6/7R did have a repeat pattern, $(GAT)_{15}$, which was visible on an autoradiograph (Fig. 28). Only 1 clone (6/12F) out of the other 15 appeared to have any type of a repeat pattern. Clone 6/12F encoded a GA-rich region of genomic DNA which contained patterns of repeats such as $(GGA)_4$GAGTGAGG, followed by a random assortment of nucleotides, then a variation of the pattern occurred

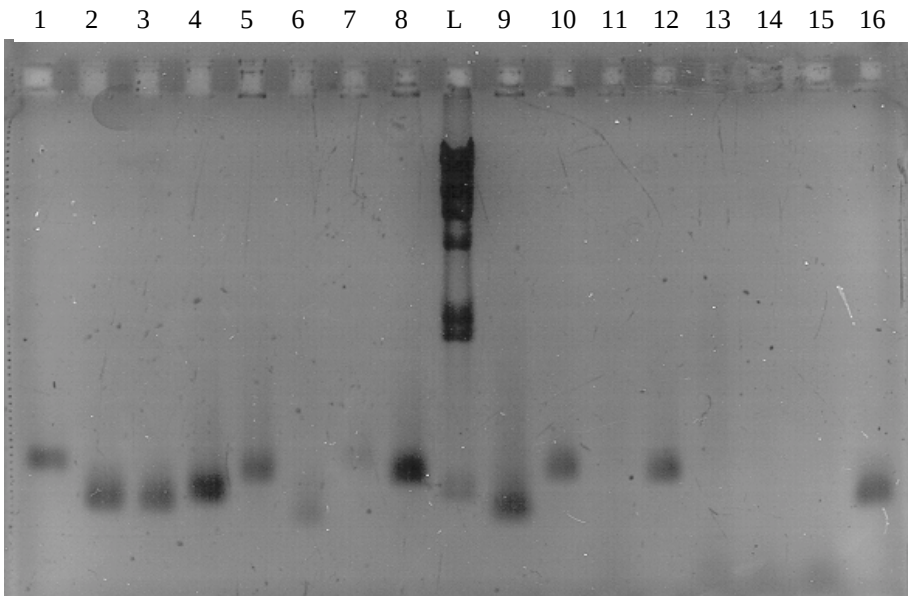

Figure 27. **Electrophoretic analysis of a 3-primer colony PCR amplification of 16 colonies isolated from the 2nd STR capture attempt.** Each of the 16 products was designated during DNA sequence reactions as 6/X, where X is the clone number (1-16). "L" designates the size standard: lambda DNA digested with *Hin*dIII.

Figure 28. **Autoradiograph of a DNA sequencing gel with the STR clone 6/7R.** This STR region, locus Aajμ1, and immediate flanking sequences have the GenBank accession #AF469474.

again: $(GGA)_3GGCAGCAGG$. Only clone 6/7R appeared to have a repeat pattern which could possibly mutate via the strand slippage phenomenon over time to yield individual Spoonbills with differently-sized products (Schlotterer and Tautz, 1992). Therefore, only that clone was chosen for oligonucleotide primer design. The DNA sequence pattern of clone 6/7R, which includes the flanking regions as well as the STR region, is shown (Fig. 29).

Two oligonucleotide primers, P7RA and P7RB, were designed to complement the flanking sequences of the repeat regions of clone 6/7R, locus Aajμ1, in order to PCR amplify the STR from genomic DNA samples. The primer sequences are as follows:

P7RA: 5'-GATCACCACCATCTTAAATGATAA-3'

P7RB: 5'-CTTCTGTTTGCCTCACATGG-3'

The design of primers for PCR amplification was facilitated by the use of the software program Primer3 (Rozen and Skaletsky, 1998). This program allowed the user to easily change design parameters. The default setting of the program allowed for the primers to have a melting temperature of approximately 60°C. The ideal product size was set to 200 bp. Each of the parameters had minimum and maximum limits of deviation which could be changed from the default settings. This allowed for various sets of primers to be designed, usually 5 sets were designed per sequence, which allowed greater choice as to primer site placement and product length (Fig. 30). The 2 PCR primers, P7RA and P7RB, were designed to amplify a product of 163 bp. Since Spoonbill #18 was used as the source of genomic DNA for this STR isolation attempt, the primers were supposed to PCR amplify a 163 bp product from Spoonbill #18, which was confirmed during the multilocus gel analysis procedure.

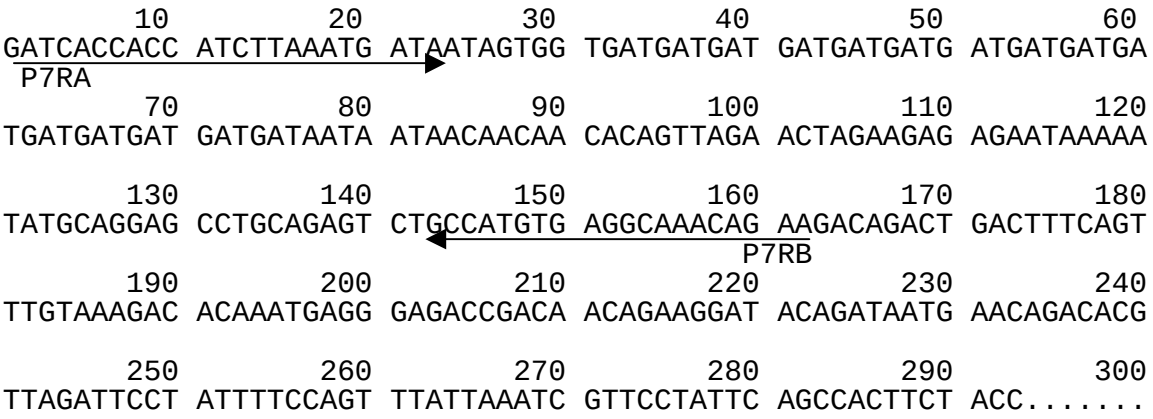

Figure 29. **Nucleotide sequence of STR locus Aajμ1.** The primers P7RA and P7RB and their relative directions are indicated by the arrows. This sequence has the GenBank accession #AF469474.

```
Primer3 Output
No mispriming library specified
Using 1-based sequence positions
OLIGO                start  len      tm     gc%   any    3' seq
LEFT PRIMER            1   24   57.11   33.33  6.00  2.00 GATCACCACCATCTTAAATGATAA
RIGHT PRIMER         163   20   59.29   50.00  4.00  2.00 CTTCTGTTTGCCTCACATGG
SEQUENCE SIZE: 201
INCLUDED REGION SIZE: 201

PRODUCT SIZE: 163, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 0.00

   1 GATCACCACCATCTTAAATGATAATAGTGGTGATGATGATGATGATGATGATGATGA
     >>>>>>>>>>>>>>>>>>>>>>>>
  61
TGATGATGATGATGATAATAATAACAACAACACAGTTAGAACTAGAAGAGAGAATAAAAA

 121
TATGCAGGAGCCTGCAGAGTCTGCCATGTGAGGCAAACAGAAGACAGACTGACTTTCAGT
                              <<<<<<<<<<<<<<<<<<<<<<<<<<<

 181 TTGTAAAGACACAAATGAGGG

KEYS (in order of precedence):
>>>>>> left primer
<<<<<< right primer

ADDITIONAL OLIGOS
                          start  len      tm     gc%   any    3' seq

 1 LEFT PRIMER          19   26   60.00   34.62  5.00  3.00 TGATAATAGTGGTGATGATGATGATG
   RIGHT PRIMER         163   20   59.29   50.00  4.00  2.00 CTTCTGTTTGCCTCACATGG
   PRODUCT SIZE: 145, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 2.00

 2 LEFT PRIMER          1   24   57.11   33.33  6.00  2.00 GATCACCACCATCTTAAATGATAA
   RIGHT PRIMER         167   21   59.07   47.62  3.00  3.00 CTGTCTTCTGTTTGCCTCACA
   PRODUCT SIZE: 167, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 1.00

 3 LEFT PRIMER          19   26   60.00   34.62  5.00  3.00 TGATAATAGTGGTGATGATGATGATG
   RIGHT PRIMER         167   21   59.07   47.62  3.00  3.00 CTGTCTTCTGTTTGCCTCACA
   PRODUCT SIZE: 149, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 3.00

 4 LEFT PRIMER          1   24   57.11   33.33  6.00  2.00 GATCACCACCATCTTAAATGATAA
   RIGHT PRIMER         156   19   60.42   52.63  4.00  3.00 TTGCCTCACATGGCAGACT
   PRODUCT SIZE: 156, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

Statistics  con   too    in    in         no    tm    tm high  high        high
              sid  many   tar  excl   bad    GC   too   too   any   3'  poly   end
            ered    Ns   get   reg   GC% clamp   low  high compl compl     X  stab    ok
Left        1020     0     0     0    21     0   790    51    59    81     0     1    17
Right        922     0     0     0     2     0   554   171    31    11     0    12   141
Pair Stats:  considered 288, unacceptable product size 108, high end compl 11, ok 169
primer3 release 0.9
```

Figure 30. **Output from the primer design program Primer3 for clone 6/7R nucleotide sequence.**

The initial work of determining if the locus was polymorphic began by amplifying 5 different Spoonbill genomic DNA samples (#1, #3, #7, #9, and #10) with the P7RA/B primer set at 58°C. The amplified products were electrophoresed on a 2% agarose gel (80 V, 1.5 hours), stained with ethidium bromide and visualized on a UV transilluminator (data not shown). The products appeared as smears at approximately 150 bp. In an attempt to better visualize the PCR products, they were loaded on a 6% (30:1) non-denaturing polyacrylamide gel, which was electrophoresed at 200 V for 5 hours (Fig. 31). Even though the precise sizes of the products was difficult to determine, it was apparent that the locus was polymorphic because of the differently-sized products.

Since more transformed (white) colonies were still available for PCR screening, 49 were amplified with the three primers, and gel electrophoresed (60 V, 2 hours on 2% agarose gels; data not shown) in an attempt to identify more STR loci. None of the samples yielded two distinct bands. Therefore, further attempts to isolate a higher percentage of STR loci, per capture cycle, was begun. The next 2 STR isolation attempts included the use of 2 different (vs. 1) biotin-labeled oligonucleotides, STR-1 and STR-2. By using 2 biotin-labeled oligonucleotides for the capture process, it was anticipated that more STR loci would be captured. From these capture attempts approximately 200 white colonies were obtained. Eighty-one of the clones were processed through the rapid plasmid preparation technique and the DNA samples were electrophoresed (80 V, 2 hours) on 1% agarose gels (data not shown). PCR amplification attempts, with 4 primers [F (-40), R (M13R), PSTR-1, and PSTR-2], on 18 of the diluted samples yielded only single bands when visualized on a 2% agarose gel (60 V for 2.5 hours).

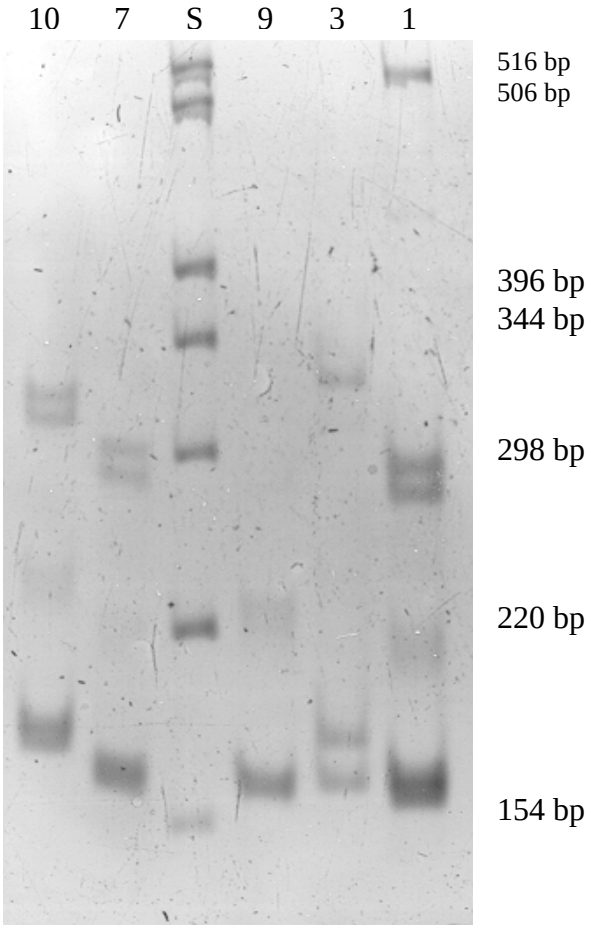

Figure 31. **Non-denaturing polyacrylamide gel analysis of Spoonbill genomic DNA samples PCR amplified with the P7RA/B primer set.** Spoonbill genomic DNA samples 1, 3, 7, 9, and 10 were amplified at 55°C. The size standard "S" is plasmid pBR322 digested with *Hin*fI. The bands nearest to the bottom are similar to the expected size of 163 bp.

The DNA sequence was determined from 20 of the samples using the reverse (M13R) and forward (-40) sequence primers. None of the sequences of the clones had a visible STR with homology to either STR-1 or STR-2. Two of the clones, C-5 and D-4F, were GA-rich and contained multiple variations on the patterns $(AAG)_4AGAA$ or $(AGG)_4CAC$. They contained an overall similarity to the clone 6/12F, in that the repeat pattern was highly degenerated relative to a perfectly repeated pattern. There was not a specific region which could be PCR amplified and be expected to yield a polymorphic profile amongst multiple Spoonbill genomic DNA samples.

Modification of the STR screening procedure

The next STR isolation attempt utilized the biotin-labeled oligonucleotide STR-3, $(GAT)_9$. Only 10 colonies (8 white, 1 blue, 1 blue and white) were obtained from this capture attempt. It appeared that the number of "background" blue colonies had dropped, or that the collection technique was not capturing even the least stringent STR clones. In order to test the ability of a radio-labeled probe to bind to the specific STR-3 [$(GAT)_9$], rapid plasmid preparations were performed on each of the 10 colonies. Approximately 2 µg of each DNA sample (designated 11/1, 11/2, 11/3, 11/4, 11/5, 11/6, 11/8, 11/10, 11/22, and 11/24) were loaded on a 1% agarose gel. Four additional samples were loaded on the gel as experimental controls: 2 µg clone 6/7R DNA, 800 ng clone 6/7R DNA, 300 ng PSTR-3 oligonucleotide DNA, and 300 ng STR-3 oligonucleotide DNA. The gel was electrophoresed at 60 V for 2.5 hours and was subsequently stained with ethidium bromide, destained in dH_2O, and then photographed (Fig. 32). The DNA was transferred to a nylon membrane (Southern, 1975) and was then hybridized with the end-labeled

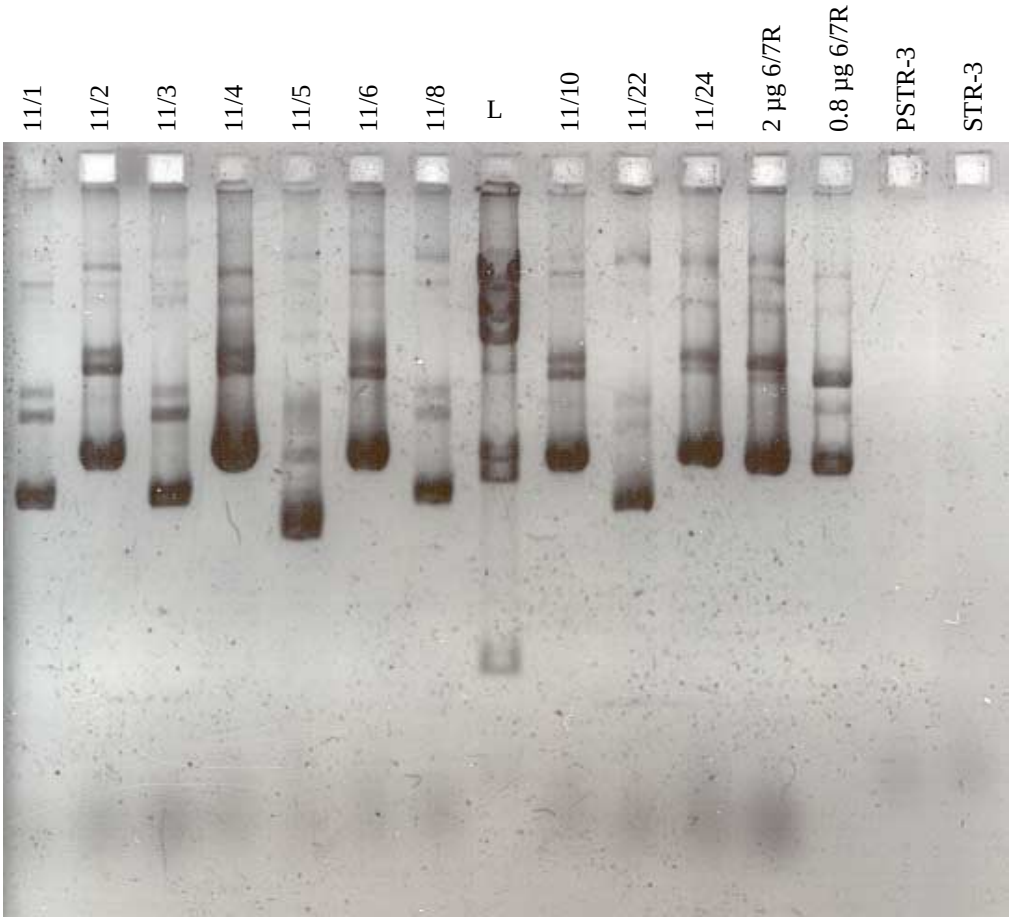

Figure 32. **Electrophoretic analysis of 11-5-99 plasmid DNA samples captured with oligonucleotide STR-3.** Plasmid DNA from the clone 6/7R (locus Aajμ1) was loaded twice, 2 µg and 0.8 µg, in addition to oligonucleotides PSTR-3 and STR-3, each at 300 ng. These samples were experimental controls when end-labeled STR-3 was used as a probe. The designated clone ID is listed above each well (same as in Fig. 33). "L" refers to the size standard: lambda DNA digested with *Hin*dIII.

probe PSTR-3. The autoradiograph showed the probe binding specifically to the 6/7R clone DNA and not to any of the most recently captured STR clones (Fig. 33). However, the successful binding of the probe to its complementary DNA on the membrane indicated the potential of using an end-labeled oligonucleotide as a probe to screen for STR containing clones among all of the white colonies created during the STR capture techniques. This was investigated and utilized further as described below.

Control experiments on the capture reagents

A series of control experiments was conducted to verify (1) the ability of the streptavidin-coated magnetic beads to bind the biotin-labeled oligonucleotides, and (2), the ability of a DNA fragment which contains an STR to bind to the biotin-labeled oligonucleotides. Two different clones, 6/4 (no repeat present) and 6/7R (contains a GAT repeat), were digested with *Afl*III and made into DNA probes by incorporating α-^{32}P dCTP as described below. Digestion of the clones with *Afl*III assisted the labeling process by creating linear fragments of the clones; clone 6/4 was cut once and clone 6/7R was cut twice. The STR primers P7RA and P7RB were used to prime the labeling reaction of 6/7R. The forward (-40) and reverse (M13R) primers of pUC18 were used to prime the labeling reaction of 6/4. Excess nucleotides were removed by passing each probe through a Sephadex® G-50 spun column.

The first control experiment began with the binding of STR-3 to the magnetic beads, followed by the hybridization (at 55°C) of DNA probe 6/4 to the STR-3/magnetic bead complex. The supernatant, collected following the hybridization registered 10,000

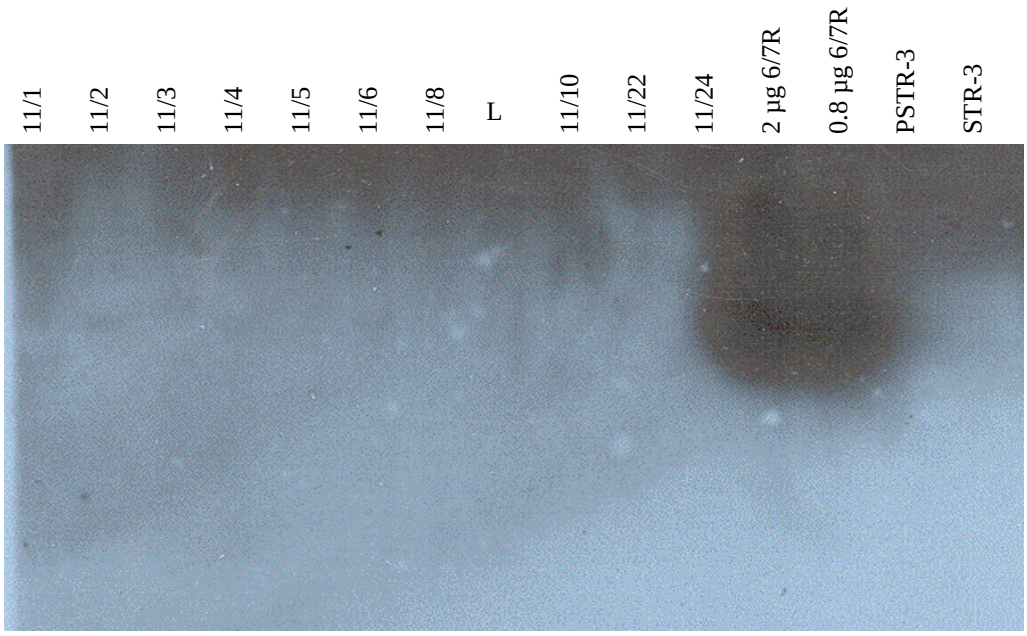

Figure 33. **Autoradiograph of Southern (1975) transferred DNA from the 11-5-99 agarose gel (Fig. 32) hybridized with the PSTR-3 probe.** The designated clone ID is listed above each well (same as in Fig. 32). "L" designates the size standard: lambda DNA digested with *Hin*dIII.

cpm with a hand-held survey meter. The first wash of the beads, to remove any residual unbound DNA probe, resulted in a supernatant containing 1,000 cpm. The 6 subsequent washes lowered the cpm in the supernatant from 150 cpm (wash #2) to 25 cpm (wash #7). Any DNA probe bound to the STR-3/magnetic bead complex was released using 20 µl of 0.15 N NaOH for 20 minutes. The eluted supernatant registered 50 cpm and the beads contained 40 cpm. Thus, the majority of the non-STR DNA probe was successfully washed from the beads. This verified the theory that the non-specific DNA probe should not bind to STR-3. Alternatively, it could still be suggested that the biotin-STR-3/streptavidin bead complex was simply not functioning properly and no DNA sequences were being captured.

The second control experiment also involved the binding of STR-3 to the magnetic beads. The DNA probe 6/7R, encoding $(GAT)_{15}/(CTA)_{15}$, was hybridized at 55°C with the STR-3/magnetic bead complex. The supernatant removed from the hybridization mixture recorded 10,000 cpm with a hand-held survey meter. The first wash of the beads to remove any residual unbound DNA probe resulted in a solution containing 4,000 cpm. The 6 subsequent washes resulted in the recovered cpm in the supernatant dropping from 80 cpm (wash #2) to 30 cpm (wash #7). Any DNA probe bound to the STR-3/magnetic bead complex was released using 20 µl of 0.15 N NaOH for 20 minutes as before. This supernatant contained 1,100 cpm, with the beads retaining 250 cpm. Since the STR DNA probe was bound to the STR/bead complex over 20 times better than the non-repeat DNA probe, this indicated the capture process appeared to be functioning properly.

The third control experiment was a repeat of the second control experiment, except that the hybridization of the DNA probe was carried out at 60°C and the washes were performed at 60°C. The supernatant from the hybridization mixture recorded 10,000 cpm with a hand-held survey meter. The first wash of the beads to remove any residual unbound DNA probe resulted in a solution containing 300 cpm. The 6 subsequent washes resulted in supernatants containing 75 cpm (wash #2) to 45 cpm (wash #7). Any DNA probe bound to the STR-3/magnetic bead complex was released using 20 µl of 0.15 N NaOH for 20 minutes. This supernatant contained 800 cpm while the beads retained 75 cpm. The STR/bead complex still captured the DNA probe relatively well, 800 cpm at 60°C versus 1,100 cpm at 55°C, at the higher hybridization and wash temperature. These results indicated that a 60°C hybridization and wash temperature, instead of 55°C, could be used in future STR-3 capture techniques and this might be expected to reduce nonspecific binding to the beads.

Additional STR isolation attempts

The next attempt to isolate STR loci began with the use of 5 different DNA templates, instead of from an individual Spoonbill (Li *et al*., 1997). The oligonucleotide STR-4, biotin-$(AAAG)_6$, was used at a hybridization temperature of 55°C, with the washes at 55°C. Only 14 white colonies, which were very small after 24 hours of growth, were created from the transformation process. The plasmids from 12 were successfully isolated and were subsequently loaded on a 1% agarose gel and electrophoresed at 100 V for 1 hour, 40 minutes (data not shown). The DNA was transferred to a nylon membrane (Southern, 1975), which was then hybridized with the end-labeled oligonucleotide PSTR-4. There was no indication on the autoradiograph that an STR locus was captured (data not shown).

Since the competent DH5α™ cells may have not been working properly (small colony size), the next isolation attempt was modified slightly, while trying to evaluate the competency of the cells. Twice as much DNA, 10 µl instead of 5 µl, from the size-fractionated and PCR amplified DNA/adapter complexes, was used during the initial capture step. The hybridization of the STR-4/bead complex occurred at 55°C, whereas the washes were performed at room temperature (23°C). A cleanup step was added to prepare the DNA for PCR amplification. The eluted DNA was passed through a G-50 Sephadex™ spun column, and then 35.5 µl was used for the next PCR amplification reaction. The remainder of the procedure was followed as outlined. Approximately 100 transformed (white) colonies were obtained.

Large scale STR screening of transformants

In order to be able to screen the large number of the transformant colonies, 64 were transferred to master plates and colony PCR amplified (forward and reverse primers only). Each of the PCR products was loaded on a 1.5% agarose gel and electrophoresed at 75 V for 2 hours. Only 43 of the chosen colonies produced a PCR product. A 1% agarose gel with 51 wells was then poured so that all 43 of the PCR amplified samples could be loaded and gel electrophoresed together at 80 V for 3 hours (Fig. 34). Unexpectantly, the PCR products from samples 21, 23 and 45 appeared to have more that one band, even though only two primers were used in the PCR amplification. These products indicate that contamination of each colony PCR with another clone may have occurred. The DNA was denatured and transferred to a nylon membrane (Southern, 1975), then affixed to the membrane by ultraviolet irradiation. The oligonucleotide PSTR-4 $(AAAG)_6$, was end-labeled with γ-^{32}P dCTP and used as a probe with this membrane. The autoradiograph produced from this screening experiment showed 5 products (2, 4, 10, 24 and 55) with some homology to $(AAAG)_6$ (Fig. 35).

The DNA sequence was determined for each of the positively hybridized DNA clones (data not shown). Only clone #10 (designated as 10R/00 during the DNA sequencing reactions) showed a promising STR repeat on the autoradiograph (Fig. 36). Clone #2 had a very short STR insert: $AAG(AAAG)_2$. Clone #(4) did not appear to have an STR insert, but did have oligonucleotide S61 inserted tandemly 6 times. Clone #24 did not have a visible insert, but did contain a region of poly-A, which was periodically interrupted with a “G” or “C”. Clone #55 encoded a degenerate STR locus: $(AAAG)_3G(AAAG)_2$. The repeat motif of clone 10R/00 consisted of $(AACT)_{13}TA(CTTT)_6CT(CTTT)_3$. The only method to determine if this locus was

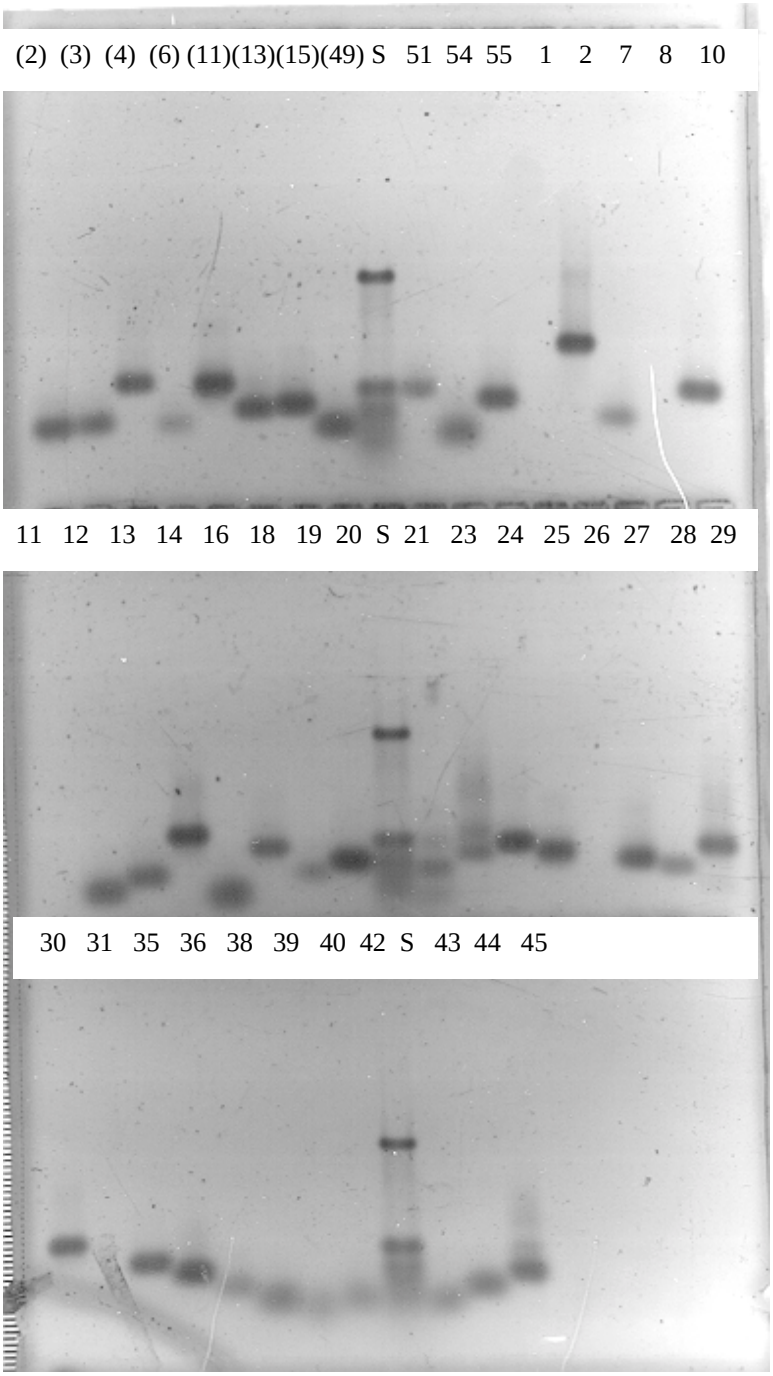

Figure 34. **Electrophoretic analysis of 43 colony PCR amplified transformants.** The identification number from each clone is indicated above the well into which it was loaded. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. This 1% agarose gel was electrophoresed at 80 V for 3 hours.

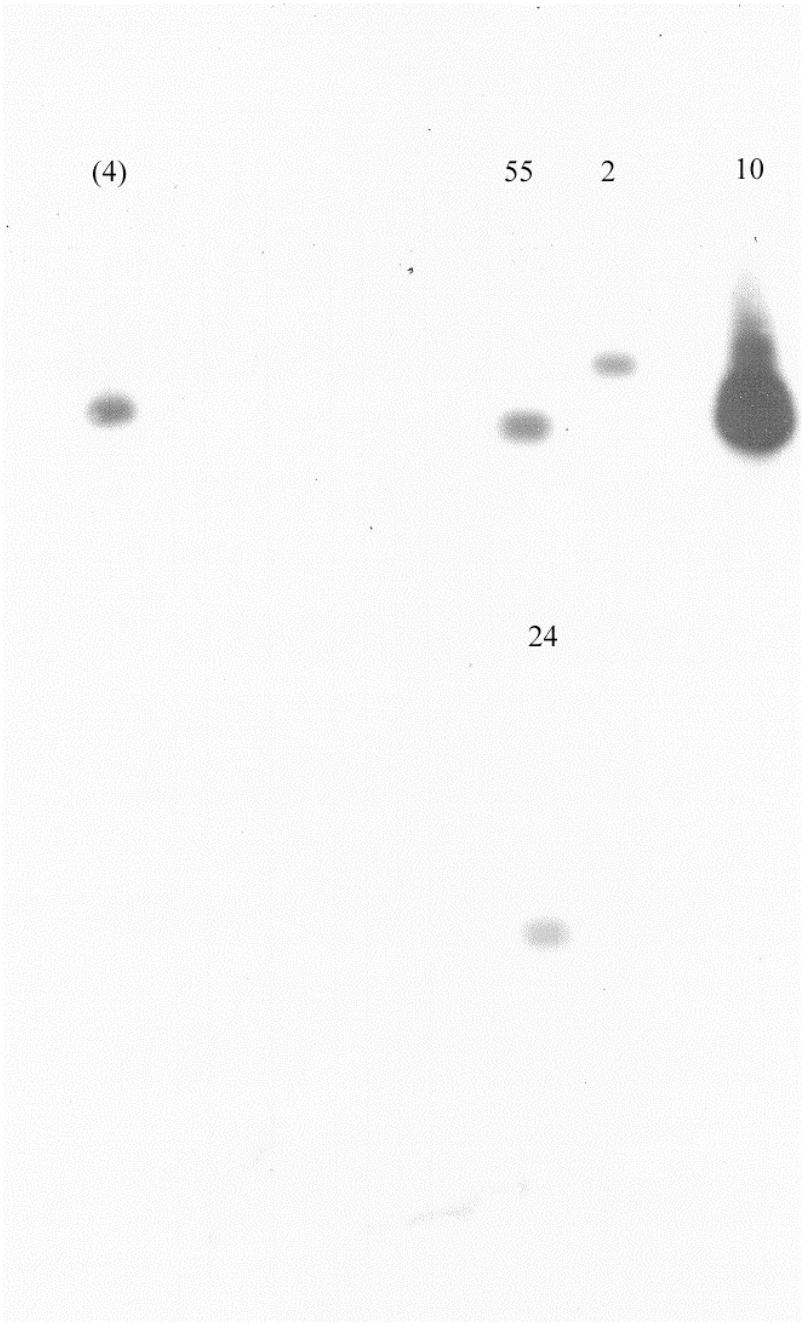

Figure 35. **Autoradiograph of the Southern (1975) transferred and PSTR-4 hybridized electrophoretic pattern created from the 43 colony PCR amplified transformants (Fig. 34).** Five amplified DNA samples [numbered 2, (4), 10, 24 and 55] have homology with the probe PSTR-4.

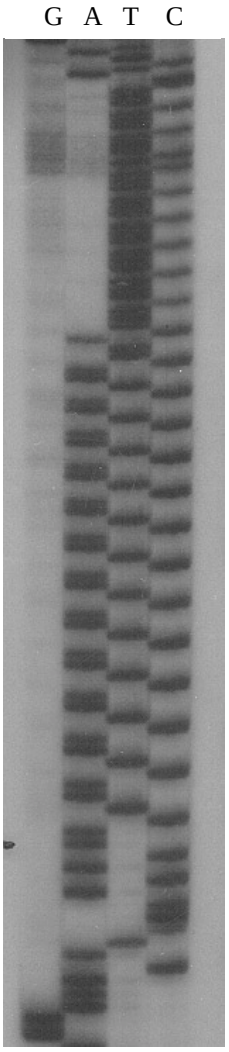

Figure 36. **Autoradiograph of the DNA sequencing gel with the STR clone 10R/00.** This STR region, locus Aajμ2, and its immediate flanking sequences have the GenBank accession #AF469475.

polymorphic among Spoonbills was to design PCR primers, with the assistance of the program Primer3 (Rozen and Skaletsky, 1998), and attempt to PCR amplify a set of Spoonbill genomic DNA samples. The flanking regions of DNA sequence were determined as far as possible from the STR (Fig. 37). Oligonucleotide primers, P10F and P10R, were then designed to PCR amplify this locus, designated Aajμ2, to create a PCR product of 217 bp. The primer sequences are:

P10F: 5'-CTTGATGCAAAGGAAACATCC-3'

P10R: 5'-GAGGTGCTTCCAGTTTCCTG-3'

It became a standard procedure to PCR amplify 6 Spoonbill genomic DNA samples every time a new primer set was designed. Multiple analyses with Spoonbills numbered 1-6 and primer set P7RA/B (data not shown), as well as the recorded pedigree, all indicated that these samples were variously unrelated, and would therefore be a useful indicator of a polymorphic locus. The primers P10F and P10R were used to PCR amplify (57°C annealing temperature) Spoonbill samples 1-6. The products were loaded on a 2% agarose gel and electrophoresed at 80 V for 1.5 hours (data not shown). The DNA bands did not appear to be the same for each sample, which indicated a polymorphic locus. Therefore, the Spoonbill samples 7-13 were then PCR amplified with primers P10F and P10R. An 8% (37.5:1) polyacrylamide gel was loaded with Spoonbill samples 1-13 and gel electrophoresed at 200 V for 5.5 hours (Fig. 38). The banding pattern visible on the gel clearly indicated a polymorphic locus by the fact there were at least 4 different band sizes.

The next STR isolation attempt (#8) was modified from the previous attempts by using 4 different oligonucleotides (STR-1, -2, -3 and -4) during the capture steps.

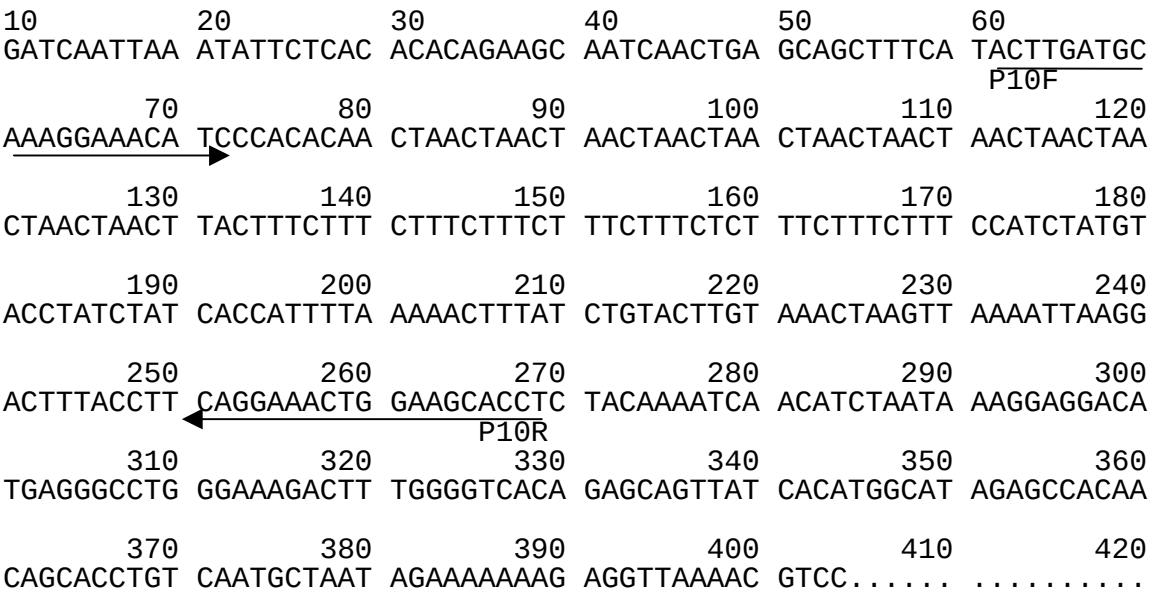

Figure 37. **Nucleotide sequence of STR locus Aajμ2.** The oligonucleotide primers P10F and P10R and their relative directions are indicated by the arrows. This sequence has the GenBank accession #AF469475.

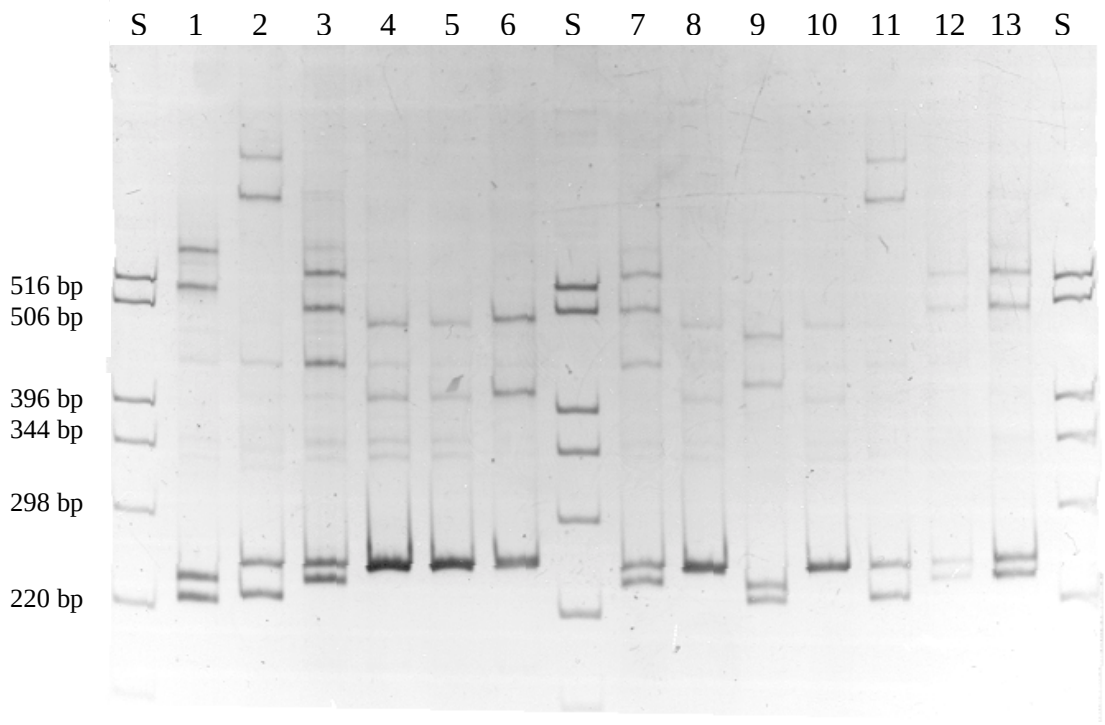

Figure 38. **Electrophoretic analysis of P10F/R amplified Spoonbill genomic DNA.** The 13 Spoonbill genomic DNA samples amplified with this primer set are indicated above each well. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. This 8% (37.5:1) polyacrylamide gel was electrophoresed at 200V for 5.5 hours.

Numerous white colonies were obtained on many of the LB-Amp^{50} plates used for the initial transformation step. Some contained only 6 white and 4 blue colonies, while others contained 70 white and 20 blue colonies. Two master plates were created, 8A and 8B, by transferring isolated white colonies from many of the plates to create these 2 plates with 50 streaks on each. Bacterial lifts using Colony/Plaque Screen™ membranes (Materials and Methods) were performed on 4 plates; 8A, 8B, 8a2 and 8b2. Two of the plates, 8a2 and 8b2, were the original plates created during the transformation process. Blue and white colonies were present on these plates. The presence of blue colonies was expected to not interfere, and they did not, with the probes binding to the transformants carrying the STRs of interest.

The oligonucleotides PSTR-1, -2, -3 and -4 were end-labeled with γ-^{32}P dCTP and used as probes with the 4 membranes. The membranes were hybridized at 55°C overnight and washed 2 times with 2.5 X SSC, 0.1% SDS, for 15 minutes, at room temperature (23°C). Each membrane registered approximately 300-500 cpm. Autoradiography was allowed to proceed for 48 hours. The images which were visible on the autoradiograph indicated the presence of 4 positive clones (clones 3/2, 3/4, 3/20 and 3/41) on master plate 8B and none on master plate 8A (Fig. 39). The colony lift for plate 82a had 5 positive light-ups: clones 3/81, 3/82, 3/83, 3/84 and 3/85. The colony lift for plate 82b had 2 positive light-ups: clones 3/86 and 3/87.

The DNA sequences of each of these clones were determined by priming each reaction with the pUC18 forward primer. Each of the clones appeared to have some type of a DNA insert with an STR. Specific analysis of each STR locus determined if the locus was polymorphic in Spoonbill genomic DNA samples. The slowest growing clone,

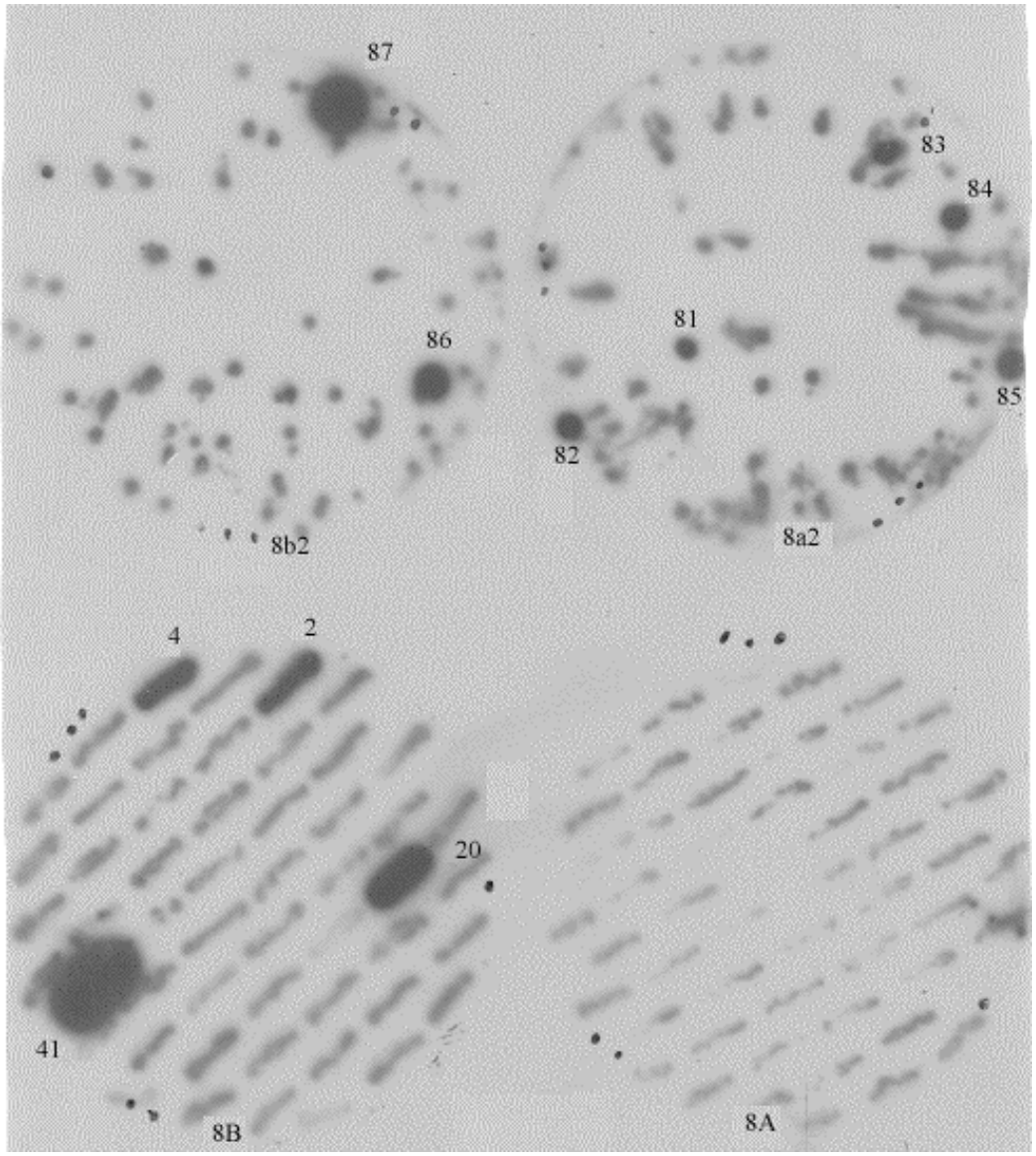

Figure 39. **Autoradiograph of 4 membranes hybridized with 4 probes: γ-^{32}P end-labeled PSTR-1, -2, -3 and -4.** Each membrane was identified at its bottom as either 8A, 8B, 8a2 or 8b2. The eleven clones selected from this experiment are numerically identified. Since these clones were identified during March 2000, they are identified in the text as clones 3/2, 3/4, 3/20, 3/41, 3/81, 3/82, 3/83, 3/84, 3/85, 3/86 and 3/87.

3/87, showed a very dark area on the autoradiograph and encoded 38 repeats of the GAT motif. However, since one side of the STR ended at the MCS, it was not possible to create a set of primers to PCR amplify this locus. Clone 3/41 was another very dark area which was visible on the autoradiograph. The transformed bacteria did grow, but very little DNA was recovered from multiple attempts of a rapid plasmid preparation. Later attempts to grow the clone met with some success. The DNA of clone 3/41 carried a $(GAT)_{17}$ with the oligo S61 on one flank and the vector near the other flank.

Clones 3/2 and 3/4 were identical and encoded the GAT motif repeated 5 times, which was probably too short to be a polymorphic locus and therefore no primers were created. Clone 3/20 encoded a 193 bp fragment with the STR motif: $(GGAT)_4GGAC(GGAT)_2$. Primers for this locus could not be created since the STR had only one flanking region (the STR ended at the MCS). Clones 3/82 and 3/84 were also identical and encoded the STR $(GGAT)_5$. The STR repeat was probably too short to be a polymorphic locus. Clones 3/83 and 3/86 each encoded a variation of the STR motif $(GAT)_5$ No primers were created for either of these clones. Sample 3/85 showed a mixed DNA sequence, with no hint of an STR, and was therefore discarded. Clone 3/81 encoded a long, but degenerated, motif: $(CCAT)_2CCA(CCAT)_3CCCA(CCAT)_3(CCGT)_3$. Nevertheless, two oligonucletide primers, P3/81A and P3/81B, were created for the flanking regions and genomic DNA samples were PCR amplified (56°C annealing temperature).

P3/81A: 5’-AATAACCCCATGCATTTTGG-3’

P3/81B: 5’-GGCTTGCAGAGCCTGATATG-3’

The expected size of the amplified product was 203 bp. The products amplified from Spoonbill genomic DNA were loaded on a 7.5% (37.5:1 acrylamide:bis-acrylamide) polyacrylamide gel and electrophoresed at 300 V for 3.5 hours. The locus did not appear to be polymorphic as a single band of the same size amplified from each DNA sample (Fig. 40).

The next attempt (#8) to capture STR regions was more successful. Therefore, the remaining DNA samples (8a and 8b) from the #8 STR capture technique were used in ligation reactions with *Bam*HI-digested pUC18 and transformed into DH5α™ (attempt #8B). Four plates of bacteria (8aC, 8aD, 8bC, 8bD) were allowed to grow overnight at 37°C. Replica plates of each were made by using Colony/Plaque Screen™ membranes. The replica membranes were hybridized at 55°C with the 4 end-labeled probes. After the wash procedure and autoradiography, 17 positively hybridized colonies (data not shown) were selected for rapid plasmid isolations.

Eight of the clones, designated 04/1, 04/2, 04/3, 04/11, 04/13, 04/14, 04/17, 04/18 and 04/19, encoded a variation of the STR motif $(GAT)_n$, where n = the number of repeats (Table VII). The number of repeats, "n", varied from 9 to 24. Some of the clones (04/1, 04/2, 04/3, 04/13 and 04/17) encoded unexpected DNA inserts, such as the S61 oligonucleotide sequence, either singularly or tandemly repeated. Since the S61 primer should have been removed during the capture technique, it is unclear as to how the primer became intergrated within these clones. However, the presence of S61 appeared to indicate a non-useful clone since one flank of the STR is either missing or includes the S61 sequence, which is not part of the Spoonbill genome.

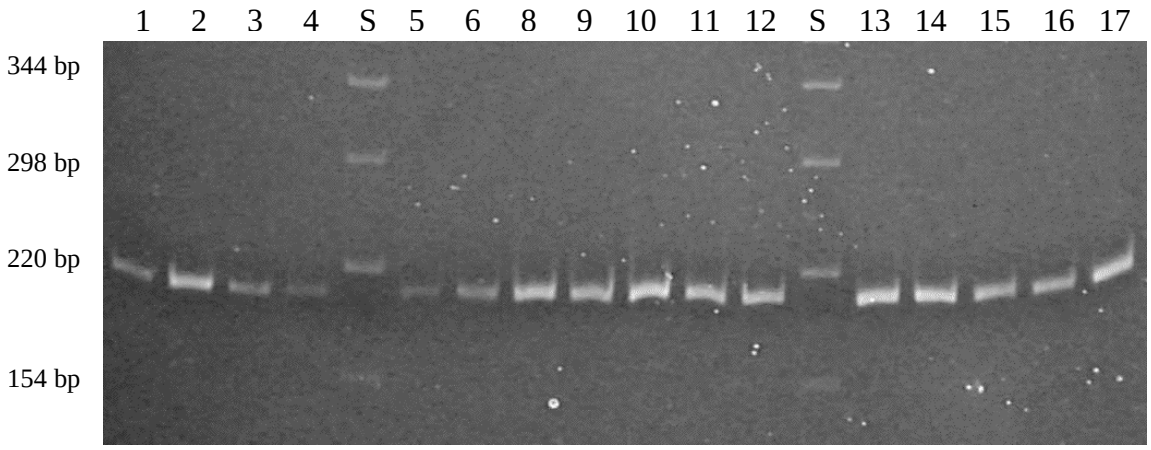

Figure 40. **Electrophoretic analysis of P3/81A/B amplified Spoonbill genomic DNA.** The Spoonbill genomic DNA samples amplified with this primer set are indicated above each well. The size standard "S" is plasmid pBR322 digested with *Hin*fI. This 7.5% (37.5:1) polyacrylamide gel was electrophoresed at 300V for 3.5 hours.

Four sets of clones were identical: clones 04/2 and 04/3, 04/4 and 04/5, 04/7 and 04/20, 04/10 and04/15. Clones 04/2 and 04/3 encoded the STR inserted at the MCS. The other 3 sets of clones encoded STR regions for which primers were designed (Table VII).

Table VII. **Analysis of clones from STR isolation #8B**			
			Amplification
Clone ID	DNA sequence	Notes	primers designed?
04/1	(GAT) x 15	STR at MCS	
04/2	(GAT) x 23	STR at MCS, same as #3	
04/3	(GAT) x 23	STR at MCS, same as #2	
04/4	$(GAT)_5GCT(GAT)_4$	Same as #5	Primers designed
04/5	$(GAT)_5GCT(GAT)_4$	Same as #4	
04/6	(GAT) x 7	STR too short	
04/7	(GAT) x 13	Same as #20	Primers designed
04/8	None	No sequence obtained	
04/10	(GGAT) x 6	Same as #15	Primers designed
04/11	(GAT) x 9	STR at MCS	
04/13	(GAT/GAC) x 19	STR at MCS	
04/14	(GAT) x 9	STR at MCS	
04/15	(GGAT) x 6	Same as #10	
04/17	(GAT) x 24	STR at MCS	
04/18	(GAT) x 9	STR at MCS	
04/19	(GAT) x 11	STR at MCS	
04/20	(GAT) x 13	Same as #7	
Problem: Primers cannot be designed when the STR is adjacent to the MCS.			

Attempts were made to amplify some of the loci, such as the 04/4 and 04/5 locus, which encoded an STR with the sequence: $(ATC)_4AGC(ATC)_5$. The oligonucleotide primers P04/5A and P04/5B were designed for this locus:

P04/5A: 5’-GGGCTCGGAACTAACTTCG-3’

P04/5B: 5’-GAACTTCTCCGACCTGGTGT-3’

The amplification at 55°C annealing temperature with these PCR primers on 7 Spoonbill genomic DNA samples was followed by the electrophoresis of the products on a 7.5% polyacrylamide gel (Fig. 41). This locus did not appear to be polymorphic since a single band of approximately 185 bp was obtained from the amplification of each sample.

Two other clones were identical; 04/7 and 04/20. These clones encoded an STR insert with the DNA sequence $(GAT)_{13}$. The DNA sequence of this locus, including its flanks, was available for primer design (Fig. 42). Two primers, P04/7A and P04/7B, were created to PCR amplify a 176 bp region of Spoonbill genomic DNA. Their sequences were:

P04/7A: 5’-CCCATGGCCACATTATAAACTT-3’

P04/7B: 5’-GCTCTGGAGTAACTTGCTGGA-3’

The primers were used to PCR amplify (55°C annealing temperature) 6 Spoonbill genomic DNA samples. The PCR products were loaded on a 2% agarose gel and electrophoresed at 80 V for 1.5 hours. An analysis of the products (data not shown) indicated the presence of one band for all samples except #14. Sample #14 appeared to have migrated slower, indicative of a slightly larger PCR product, than all of the other samples. Since this result was indicative of a possible polymorphic locus, 16 Spoonbill genomic DNA samples were PCR amplified, as above. The products were loaded on a 7.5% polyacrylamide gel and electrophoresed at 250 V for 4.5 hours (Fig. 43). The STR locus 04-7 was definitely polymorphic since two distinctly different PCR amplified products were visible on the polyacrylamide gel.

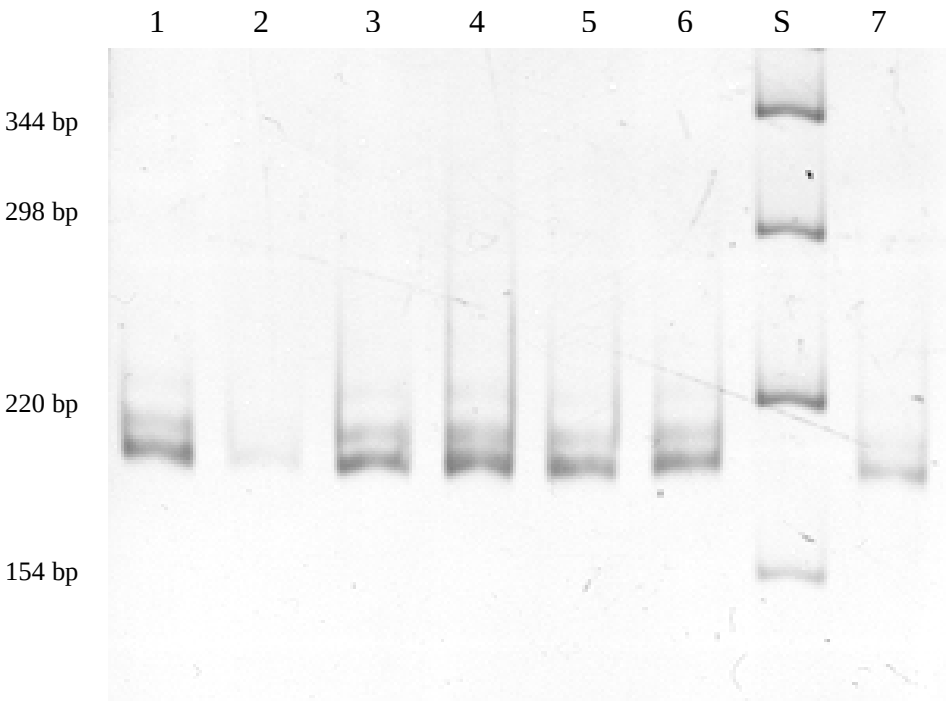

Figure 41. **Electrophoretic analysis of P04/5A/B amplified Spoonbill genomic DNA.** The 7 Spoonbill genomic DNA samples amplified with this primer set are indicated above each well. The size standard "S" is plasmid pBR322 digested with *Hin*fI. This 7.5% (37.5:1) polyacrylamide gel was electrophoresed at 100V for 10 hours.

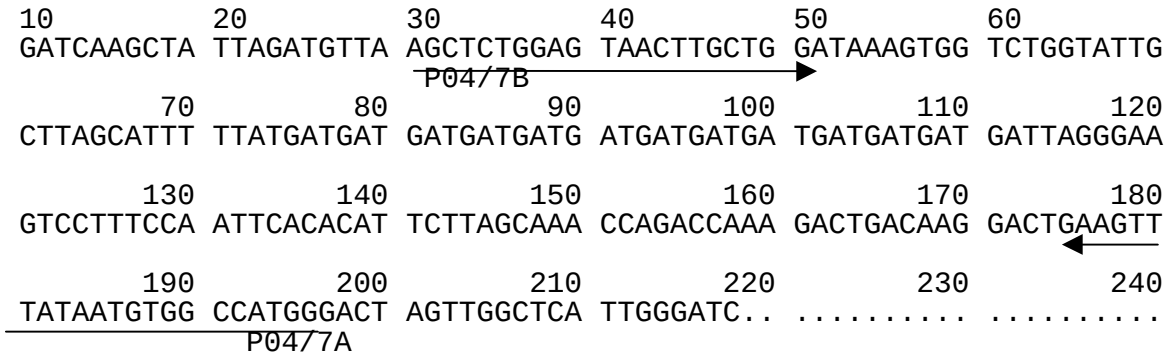

Figure 42. **Nucleotide sequence of STR locus Aajμ3.** The oligonucleotide primers P04/7A and P04/7B and their relative directions are indicated by the arrows. This sequence has the GenBank accession #AF469476

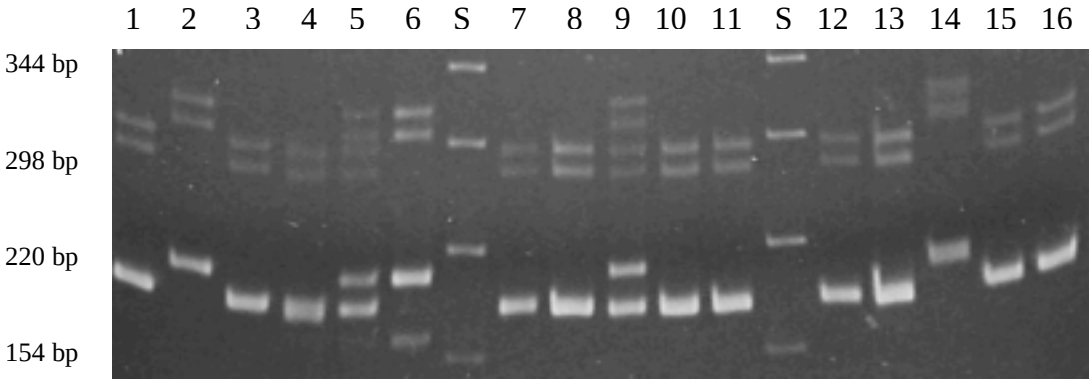

Figure 43. **Electrophoretic analysis of P04/7A/B amplified products of Spoonbill genomic DNA.** The 16 Spoonbill genomic DNA samples amplified with this primer set are indicated above each well. The size standard "S" is plasmid pBR322 digested with *Hin*fI. This 7.5% (30:1) polyacrylamide gel was electrophoresed at 250V for 4.5 hours.

A third set of PCR primers, P04/10A and P04/10B, were designed to amplify a 274 bp product from the locus of clones P04/10 and P04/15. The primers were designed as follows:

P04/10A: 5'-TCCAGCCTCCTTTCAGGTAG-3'

P04/10B: 5'-CTTACCCTTCCCCATCCATT-3'

The amplification of Spoonbill genomic DNA with these primers occurred at 58°C annealing temperature. Each of the samples appeared (2% agarose gel analysis) to have produced a single band at approximately 260 bp. A 7.5% (37.5:1) polyacrylamide gel analysis of the products showed the same bands were created from each sample, which is indicative of a non-polymorphic locus (Fig. 44).

The next attempt (#9) to capture STR loci was almost the same as attempt #8, except for the following changes. Size-selected DNA (10 µl) from a previous STR isolation attempt (11/18/99) was used. Most importantly, STRs-5, 6, 7, and 8 (100 pmol of each) were used in the capture procedure. Five master plates, 9A, 9B, 9C, 9D and 9E, were created with approximately 50 streaks on each from the transformed colonies. After the master plates were incubated overnight at 37°C, portions of the colonies were transferred to Colony/Plaque Screen™ membranes. The DNA was denatured and affixed to each as directed (Dupont NEN™), and then the lifts were hybridized at 55°C overnight with 2 million cpm of a STRs-5/6 probe mixture and 1 million cpm of a STRs-7/8 probe mixture. The membranes were washed at room temperature (23°C) for 15 minutes in 2.5X SSC, 0.1% SDS. This wash was repeated. Each membrane was surveyed at approximately 400-500 cpm. An autoradiograph (2 days) was performed on the 5

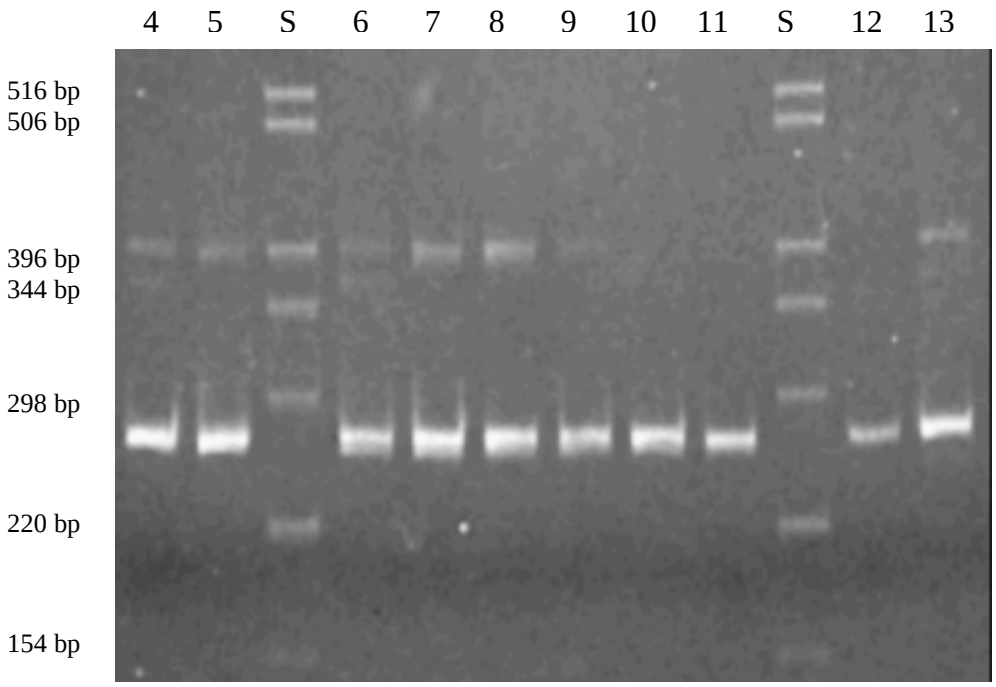

Figure 44. **Electrophoretic analysis of P04/10A/B amplified products of Spoonbill genomic DNA.** The 10 Spoonbill genomic DNA samples amplified with this primer set are indicated above each well. The size standard "S" is plasmid pBR322 digested with *Hin*fI. This 7.5% (37.5:1) polyacrylamide gel was electrophoresed at 250V for 4.5 hours.

membranes (data not shown). Every streak on the master plate appeared to be positively hybridized. In order to increase the stringency of probe binding, the membranes were washed again at 55°C for 15 minutes. A second autoradiograph of these washed membranes assisted in the identification of 16 positively hybridized bacterial streaks (data not shown).

Each of the 16 bacterial streaks was added to separate 5 ml LB broth tubes, which were supplemented with 50 µg/µl ampicillin. Only 12 of the bacterial samples grew. This set of 12 was processed through a rapid plasmid procedure and 2 µg samples were loaded on a 1% agarose gel. The gel was electrophoresed at 100 V for 2 hours, then stained with ethidium bromide and photographed to check for the presence of plasmid DNA (data not shown). Each of the samples contained plasmid DNA. Therefore, the DNA sequence (forward and reverse primers) of each clone was determined. The characteristics of each clone are outlined below (Table VIII).

Two clones were identical; 9/6 and 9/15. Primers were designed to PCR amplify this locus. Initially, the presence of the *Sau*3AI restriction endonuclease cutting site was not noticed and primers were created on the wrong side of this site. The first set of primers, 9-6A and 9-6B, did not result in a PCR amplified product from Spoonbill genomic DNA. Further modifications of the primers to create 9-6C and 9-6D also did not yield a PCR amplified product. One more primer, 9-6E, was designed:

9-6D: 5’-GATCCAGTTGGTGAGGAGATGC-3’

9-6E: 5’-AGAAAGAAGGGTGTGCCTCA-3’

Table VIII. **Analysis of clones from STR isolation #9**			
			Amplification
Clone ID	DNA sequence	Notes	primers designed?
9/1	(AGG) x 12	STR at MCS	
9/2	(AGG) x 8	STR at MCS, same as #3	
9/3	(AGG) x 8	STR at MCS, same as #2	
9/4	(AGG) x 8	STR at MCS	
9/5	(AGG) x 12	STR at MCS	
9/6	(AGG) x 12	short (102 bp) clone, same as #15	Primers designed
9/9	(AGG) x 7	STR too short and some sequence unreadable	
9/12	(TGG) x 6	STR at restriction site	
9/13	(AGG) x 11	STR at MCS	
9/14		No visible STR	
9/15	(AGG) x 12	short (102 bp) clone, same as #6	
9/16		No visible STR	
Problem: Primers cannot be designed when the STR is at the MCS.			

The expected product size from PCR amplification with 9-6D and 9-6E was 102 bp. The PCR amplification of genomic DNA at 58°C annealing temperature did produce a very small DNA product with some Spoonbill genomic DNA samples (2% agarose gel analysis). Amplifications were also performed at 57 and 60°C, which resulted in no discernable differences between the products. The 58°C amplified products were loaded on a 7.5% (37.5:1) polyacrylamide gel and electrophoresed at 100 V for 10 hours (Fig. 45). Products were visible between 160 and 180 bp and between 220 and 230 bp, but were not visible at 100 bp. The same bands appeared to be present in each sample. Therefore, this locus was considered to be non-polymorphic.

The next STR isolation attempt (#10) was slightly modified from #9. A different set of Spoonbill genomic DNA samples (8, 10, 17, 18 and 22) was digested with *Sau*3AI.

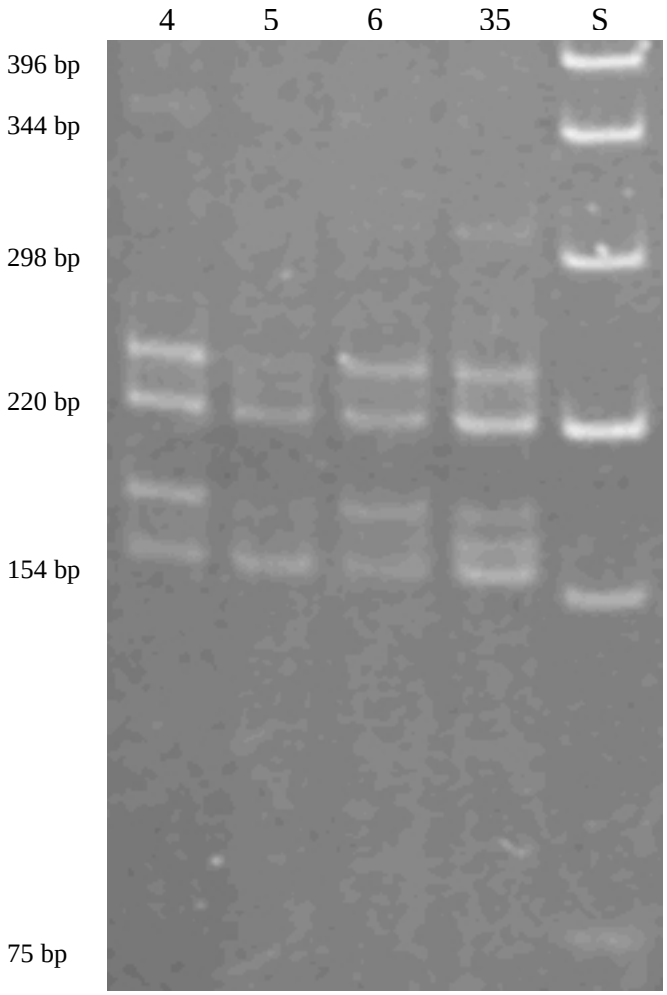

Figure 45. **Electrophoretic analysis of 9-6D/E amplified Spoonbill genomic DNA.** The 4 Spoonbill samples amplified with this primer set are indicated above each well. The size standard "S" is plasmid pBR322 digested with *Hin*fI. This 7.5% (37.5:1) polyacrylamide gel was electrophoresed at 100V for 10 hours.

Also, the method of size-selecting DNA (300-800 bp) from an agarose gel was changed from using the Wizard Prep® DNA isolation kit (Promega) to using Gelase™ (Epicentre®). Finally, the vector was changed to pBluescript™. Six agar plates of bacteria were created from the transformed cells. Approximately 50 blue and 50 white colonies were present on each plate. A separate Colony/Plaque Screen™ membrane was used on each of the agar plates. After the membranes were properly treated to denature and affix the DNA, they were hybridized overnight at 55°C. The membranes were washed at room temperature (23°C) for 15 minutes in 2.5 X SSC, 0.1% SDS. The wash was repeated. A survey of the membranes showed 300-400 cpm. An autoradiograph was performed for 14 hours (data not shown).

It was difficult to identify the colonies which appeared to have been positively hybridized, primarily because the lift procedure, which used the Colony/Plaque Screen™ membranes, often left the bacterial colonies smeared or removed them totally from the plate. It appeared the Colony/Plaque Screen™ membranes should be used on relatively dry plates. Seven colonies were chosen and processed through a rapid plasmid isolation procedure. The DNA sequence from each of these samples was determined by using the universal forward and reverse primers of pUC18.

The clones SB4, SB5 and SB6 each encoded an STR. Clone SB4 encoded the STR $(TGG)_7$, but it was closely flanked by the *Sau*3AI sites, which rendered it useless. Clone SB5 encoded the STR $(TGG)_9$, which was also flanked by *Sau*3AI sites. Clone SB6 encoded the STR $(CCT)_{11}$, which was flanked on one side by a *Sau*3AI site. None of the other colonies appeared to have an STR insert.

Modification of the oligonucleotide adapter

During these latest STR isolation attempts, I had isolated 12 STR loci from flamingo genomic DNA and determined the DNA sequence from each clone. Nine of these clones contained the same critical problem found in some of the Spoonbill STR clones. The critical problem was the location of the STR locus (with multiple repeats) adjacent to the clone's MCS. Therefore, I needed to further modify the STR isolation procedure in order to better optimize the capture technique. Many of the STR loci appeared to have the expected restriction endonuclease site *Sau*3AI, although sometimes it did appear to be truncated to GAT. There were an inordinate number of *Sau*3AI sites in close proximity to the STR loci which were captured. Attempts were made to circumvent this problem by designing 2 new types of oligonucleotide adapters: *Tsp*509I, using the S63/S61 combination, and *Hin*P1I, using the S64/S61 combination (see Materials and Methods).

The next STR isolation procedure (#11) began with the digestion of 5 different Spoonbill samples (1 µg each) with *Tsp*509I. The DNA was size-fractionated to 200-1500 bp and purified by the Wizard Prep™ DNA isolation method (Promega®). The S63 was annealed to S61 and ligated to the size-fractionated DNA. Two different mixtures of oligonucleotides were used in the capture events. STRs -1, 2, 3 and 4 were used in one capture procedure. STRs- 5, 6, 7 and 8 were used in another capture procedure. Both procedures followed the outline as in Materials and Methods, except using *Tsp*509I whenever *Sau*3AI was specified. Two master plates, 11A and 11B, were created from the transformed colonies. Colony/Plaque Screen™ lifts were performed on these plates and then they were hybridized with PSTR probes 5/6 and 7/8. The

membranes were hybridized with the probes overnight at 55°C and then washed at room temperature (23°C) as before. An additional wash was performed at 55°C for 10 minutes to reduce the cpm on each membrane. Every colony on the autoradiograph (16 hours) was positively-hybridized. A subsequent wash at 60°C, followed by autoradiography, still did not allow differentiation of STR-containing clones. It appeared as though one of the probes was binding non-specifically and strong, which obscured the ability to select the appropriate colonies.

Two more master plates, 11C and 11D, were created from transformants of the latest STR isolation procedure. 11C was hybridized overnight at 55°C with PSTR probes 1/2 and 3/4. 11D was hybridized overnight at 55°C with PSTR probes 5/6 and 7/8. Each membrane was washed separately at room temperature (23°C). 11C exhibited 400-500 cpm. 11D exhibited between 500 and 1000 cpm. Autoradiographs of both membranes indicated the binding of the probes was non-selective. All of the bacterial streaks were hybridized. However, none of the probes appeared to have an exceptionally strong affinity for any specific clone. Therefore, 4 white colonies were chosen at random and the nucleotide sequence of each was determined. There was no visible STR within any of the clones.

The next STR isolation attempt (#12) used twice the amount of magnetic beads, 200 µl instead of 100 µl. STRs-3, 6, 7, and 8 were used to capture the STR clones since each oligonucleotide consisted of trinucleotide repeats. The same Spoonbill DNA which was digested with *Tsp*509I, ligated to the adapter, and then PCR amplified for isolation #11, was used in this attempt. The hybridization of DNA fragments to beads occurred at 50°C instead of at the normal 55°C. The transformation results varied from 4 blue

colonies and 2 white on one agar plate to 20 blue colonies and 16 white on the other agar plate. The total number of white colonies was considerably less than expected (50-100).

Reagents assay

Control experiments were then performed to test the efficiency of specific reagents. Five Spoonbill DNA samples (1 µg each) were digested with *Tsp*509I , and five separate Spoonbill DNA samples (1 µg each) were digested with *Hin*P1I. Each digestion was electrophoresed on a gel and the DNA was size-fractionated (300-800 bp) and purified by the use of Gelase™. The purified DNA was then placed in a ligation reaction with an appropriately digested vector. *Tsp*509I-digested DNA was ligated to *Eco*RI-digested vector. *Hin*P1I-digested DNA was ligated to *Cla*I-digested vector. Portions of each ligation (10 µl out of 20 µl) were transformed into competent DH5α and plated onto LB/Amp^{50} plates. One plate had no growth and the other plates had only 1-8 colonies on them. Apparently, the digested DNA fragments did not ligate properly to the vectors, which prevented the transformation of competent cells.

The vector pUC19 was digested with *Hin*P1I. The digested DNA was then mixed with competent cells and plated. This resulted in no growth of colonies, which was expected. The *Hin*P1I-digested vector was then ligated to *Cla*I-digested (and alkaline phosphatase treated) pBluescript® using (1) GIBCO® ligase and buffer, and (2) GIBCO® ligase and freshly made ligase buffer (ATP and DTT added separately). Transformation experiments with portions of these ligation products resulted in both agar plates, (1) and (2), containing approximately 200 white colonies and 20 blue colonies. A transformation with just the *Cla*I-digested (and alkaline phosphatase treated) pBluescript® resulted in

only one blue colony on an agar plate. The GIBCO® ligase, when used with either buffer, proved to work efficiently. The restriction endonuclease digestions of the target and vector DNAs appeared to be work appropriately as well.

Use of the modified adapters

Two new STR isolations were begun with the ligation of the S64/S61 adapter to the *Hin*P1I-digested Spoonbill DNA and the ligation of S62/S61 to *Sau*3AI-digested Spoonbill DNA. After the DNA samples were ligated, a 5 µl portion of each was PCR amplified, with S61 as the primer (as per the normal procedure). A 5 µl portion of each PCR amplified sample was then loaded on a 2% agarose gel and electrophoresed at 80 V for 1 hour. Only PCR artifacts, i.e. primer dimers, were evident within the gel. The PCR was performed again with new *Taq* DNA polymerase (Fisher) and the annealing temperature was raised to 60°C for the *Sau*3AI-digested DNA, and 57°C for the *Hin*P1I-digested DNA. A portion of each of these samples (5 µl) was electrophoresed on a 2% gel (80 V for 1 hour). Each one showed a distinct smear from 50 to 600 bp, as expected for the size-fractionated and PCR amplified DNAs.

The remainder of the procedure was followed as outlined in Materials and Methods. However, the captured DNA was ligated into 2 different vectors, pBluescript® and pUC19, which were digested with the appropriate restriction endonuclease, in order to ascertain if there is a difference in vector quality relative to cloning efficiency. From 8 plates of transformed cells (4 were pBluescript® and 4 were pUC19), the *Sau*3AI ligation only yielded 5 white and 25 blue colonies total. From the 4 plates of transformed cells (all pBluescript®), the *Hin*P1I ligation yielded about 85 white and 130 blue colonies. The

results of the *Sau*3AI ligation appear to support the notion that neither vector was better prepared than the other. However, the DNA from the *Hin*P1I ligation did, for some unknown reason, ligate more efficiently.

Additional amounts of the captured DNA were processed through the latest method of STR isolation and transformation. The white colonies from the *Sau*3AI and *Hin*P1I ligations were transferred to master plates; [1] STRs 1-4, [2] STRs 1-4, A1 12/7/00 STR 1-4, [3] STRs 5-8, [4] STRs 5-8 and B2 12/7/00 STRs 5-8. At this point, the master plates were created in duplicate, which enabled one plate to be sacrificed during the lifting procedure and still have the other plate from which to choose a specifically hybridized bacterial streak. The membranes which contained colonies that were captured with STRs 1-4, were hybridized overnight at 55°C with the PSTR-probes 1/2 and 3/4. The membranes which contained colonies that were captured with STRs 5-8, were hybridized overnight at 55°C with the PSTR-probes 5/6 and 7/8. All of the membranes were washed, as above, and exposed to film. An autoradiograph (48 hour exposure) revealed some probe specificity on only 6 of the master plate samples. The plasmid DNA was isolated from 2 of these samples, 12-4 and 12-6. Both of these were subjected to DNA sequence analysis. Clone 12-4 did not provide a readable sequence (no DNA profile was visible) and clone 12-6 encoded an STR, $(AGG)_{12}$, which was adjacent to the MCS.

From these results, it appeared the STR capture technique had become very inefficient, or perhaps too stringent. Neither clone from the latest screening procedure appeared to be useful. To ensure the magnetic beads, as well as the other reagents, still

worked, the second control experiment using the STR-containing clone (6/7R) was repeated. As before, every reagent appeared to be working as expected.

Since all of the reagents appeared to be working properly, additional efforts were applied to screen as many colonies as possible for the presence of STR loci. Four additional master plates, Δ1, Δ2, Δ3 and Δ4, were created from the latest *Hin*P1I capture technique. A Colony/Plaque Screen™ membrane was used on each of these plates and subsequently prepared for hybridization. Membranes Δ1 and Δ2 were hybridized overnight at 55°C with the 4 probes PSTR-1, 2, 3 and 4. Membranes Δ3 and Δ4 were hybridized overnight at 55°C with the 4 probes PSTR-5, 6, 7 and 8. The membranes were washed at room temperature (23°) 2 times and then exposed to X-ray film. The autoradiograph showed 4 areas on membrane Δ4 which appeared to bind a probe better than the other colonies. Each of these four clones was processed through a DNA sequencing procedure. Clone 12C19 appeared to carry an (AGG) repeat. The area was compressed on the autoradiograph, but the STR did appear to be cloned adjacent to the MCS, which rendered it useless as a polymorphic locus. Clone 12C20 encoded the STR $(AGC)_4(AGG)_9$, which was also inserted at the MCS. Clone 12C22 did not appear to have an STR insert. The 4th clone, 12C28, did not produce a DNA sequence on the autoradiograph.

The remaining DNA samples from the latest STR isolation attempts were ligated to an appropriately digested vector and transformed into competent DH5α™ and spread onto LB-Amp^{50} plates. Each of these plates [(5), (6), (7), (8), (9) and (10)] contained approximately 25-100 white colonies and 25-100 blue colonies. Since the creation of master plates with all of these colonies would be extremely tedious, each of the plates

was replica plated to create "A" and "B" lifts by the use of Colony/Plaque Screen™ membranes. The agar plates had been thoroughly pre-dried before the addition of the transformed cells. This step helped prevent the disruption (smearing) of the colonies during the replica plating procedure. All of the Colony/Plaque Screen™ lifts were treated to denature and affix the DNA. The membranes from Δ5, Δ6, and Δ9 were hybridized overnight at 55°C with the probes PSTR 1/2 and 3/4. The membranes from Δ7, Δ8, and Δ10 were hybridized overnight at 55°C with the probes PSTR 5/6 and 7/8.

All of the membranes were washed at room temperature (23°C) 2 times before exposing them to X-ray film. After a 48-hour exposure, the film was developed. There were no positive marks on the membranes hybridized with the probes PSTR 1/2 and 3/4. There were some positively-hybridized areas on some of the membranes hybridized with the probes PSTR 5/6 and 7/8. Each of these areas was aligned with bacterial colonies from the original plates as best as possible. The selected colonies were then transferred to a master plate: Master 1-3-01. A colony lift was performed on the master plate by using a Colony/Plaque Screen™ membrane, which was subsequently processed for overnight hybridization at 55° with probes PSTR 5/6 and 7/8. Six of the clones were chosen for DNA sequence determination. Clone SB11 did not contain an STR insert. Clone SB21 was approximately 80 bp and encoded the STR $(AGG)_8$. The size of the DNA insert, 80 bp, and the presence of only 8 trinucleotide repeats, made this site highly undesirable as a candidate for the design of a specific PCR primer set.

Two clones, SB12 and SB14, were identical. They also encoded a large STR region: $(AGC)_{18}$. Two other clones, SB19 and SB24, encoded the same type of STR, only shorter: SB19 had $(AGC)_{15}$, and SB24 had $(AGC)_9$. It is noteworthy that all 4

clones had one identical DNA flanking sequence. However, the 3 types of clones did have 1 flanking sequence which was different. The STR of clone SB19 mutated from $(AGC)_{15}$ to $(AGG)_7$, which was then flanked by a unique sequence. The STR of clone SB24 mutated from $(AGC)_9$ to an (ACC)-rich region of 77 nucleotides, which appeared to be the degenerated form of $(AGC)_n$.

Multiple oligonucleotide primers (12A, B, C, D, E, 19A and B) were designed to PCR amplify these loci from Spoonbill genomic DNA. Primer 12B was accidentally designed within the vector DNA sequence. Therefore, the PCR amplifications with primer sets 12A/B and 19A/B did not yield a specific DNA product. Primer 12C was made to replace 12B. Multiple attempts to PCR amplify Spoonbill genomic DNA samples with primer 12C, at either the 57° or 63°C annealing temperature, resulted in products too small, about 70 bp for 12A/C, or too large, about 800 bp for 19A/12C. A new primer, 12D, was designed to bind just a few nucleotides downstream of 12C, in an attempt to better prime the locus. PCR amplifications with 12A/D and 19A/12D on Spoonbill genomic DNA at 57, 60 or 63°C, did not yield a specific PCR product.

The DNA sequence of clone SB12 (183 bp) was compared to the GenBank database by the use of BLASTN 2.2.1 (Altschul *et al*., 1997). The first 27 nucleotides of the sequence (5'-CGCCTTAGACCGCTCGGCCATCCTGAC-3') showed perfect homology (100% match: 27/27 nucleotides) with 21 regions of DNA files. An additional 45 regions of DNA files had only one nucleotide mismatched, for a homology of 96% (26/27 or 27/28 nucleotides). Some of the files, which encoded homologous regions, are listed as genomic scaffolds (*Drosophila melanogaster*) or transfer RNA (tRNA) genes. It is unclear as to the purpose, if any, of this region. However, since its presence may have

been a cloning artifact, instead of the exact sequence at that locus, the primer 12E was designed directly downstream of primer 12D. Primer 19B was also designed downstream of 19A in order to avoid a *Hin*P1I restriction site. The primers are:

12D: 5'-GAGGGAGAGCAGCAGCAG-3'

12E: 5'-ACCACTGCTGCTTCTGCTG-3'

19B: 5'-CCTCTCCTTGCCTCAACG-3'

The amplification of Spoonbill genomic DNA with the primer set 12D/E at a 60°C annealing temperature yielded only small products, less than 100 bp, which were most likely non-specific PCR artifacts such as primer dimers. This conclusion was confirmed when the same products were not visible on a polyacrylamide gel (7.5%). The amplification of Spoonbill genomic DNA with the primer set 19B/12D at 55°C annealing temperature yielded faint bands about 150 bp and distinct bands about 360 bp for Spoonbills #1-#6 and #15. A distinct band about 470 bp was observed from samples #13 and #14. These products were visible on a 7.5% polyacrylamide gel (Fig. 46). Subsequent attempts to preferentially amplify the 470 bp band, instead of the 360 bp band, were unsuccessful. The 360 bp band appears to be the preferentially amplified product while the 470 bp product weakly expressed. The amplification at 50°C annealing temperature of Spoonbill genomic DNA (samples #2-#5 and #10-#15) resulted in a distinct band about 360 bp and 2 faint bands about 150 bp (7.5% polyacrylamide gel, data not shown). The amplification of much larger products (360-500 bp instead of 100 bp products) from Spoonbill genomic DNA, and the uniformity of the amplified products at 50°C, supported the designation of these loci as non-polymorphic.

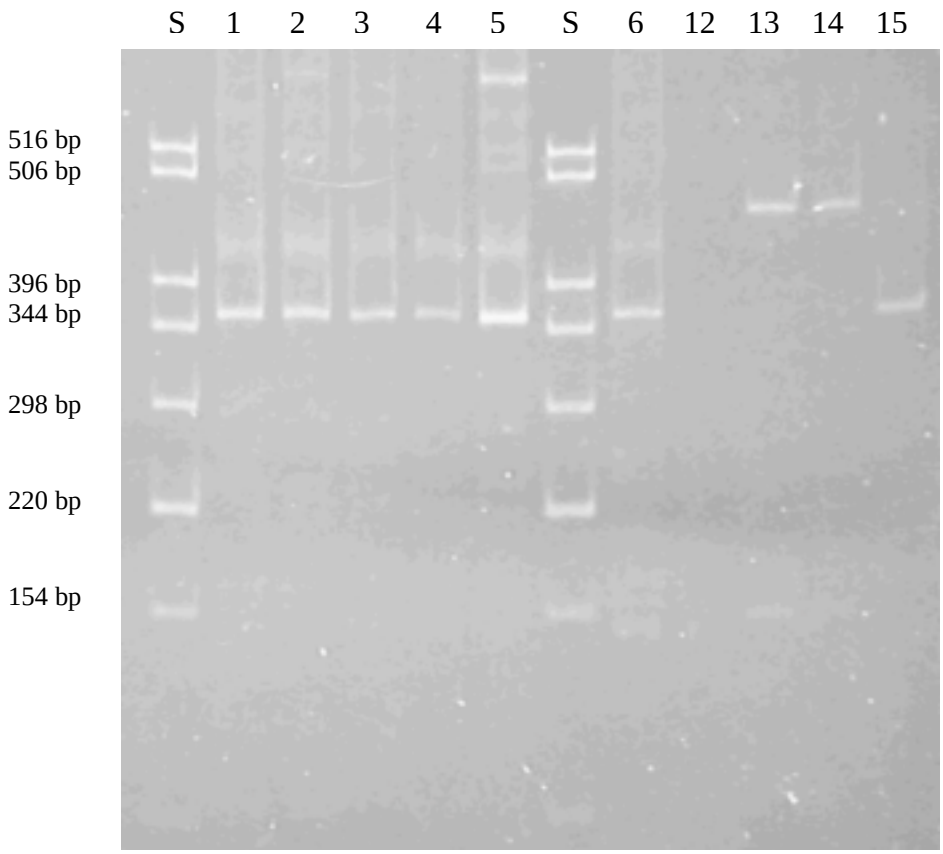

Figure 46. **Electrophoretic analysis of 12D/19B amplified Spoonbill genomic DNA.** The Spoonbill genomic DNA samples were amplified at 55° annealing temperature and are indicated above each well. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. This gel was 7.5% (37.5:1) polyacrylamide which was electrophoresed at 100V for 10 hours.

The next STR isolation attempt began with the *Hin*P1I digestion of 5 µg Spoonbill genomic DNA (1 µg from 5 different samples). The fraction of DNA consisting of 200-800 bp was removed from an agarose gel as directed in Materials and Methods. The adapter S64/S61 was then ligated to the size-fractionated DNA. The capture attempt was performed at 45°C with STRs -6, 8, 11 and 12. The captured DNA was then ligated to the vector DNA and transformed to create 12 agar plates. A lift was performed on each of the plates by using the Colony/Plaque Screen™ membranes and each was properly treated to denature and affix the DNA. All the membranes were hybridized at 55°C overnight with the STR probes 6/8 and 11/12. The membranes were exposed for 8 hours to the X-ray film. The average number of positively-hybridized colony light-ups was 5 per membrane. One membrane only had 1, while another had 9.

The rapid plasmid isolation procedure was performed on 9 isolated colonies. The DNA sequence from each of these was determined. Clones A2 and B6 were identical, as well as B1, B2 and B3. Every clone encoded STR regions. The STR $(AGG)_n$, where n = 7-9, was present in each of them, and there were usually multiple sites of this STR, or at least variations of it. However, the distinct STR repeat $(AGG)_n$ was always directly adjacent to the MCS, and therefore could not be amplified from the genome.

Plasmid DNA isolations were performed on an additional 9 samples. Each of these was processed via the DNA sequencing procedure. Clones 17H and 22H did not produce a profile on the autoradiograph. Clone 7H encoded the STR repeat $(CAA)_6$, which was considered too short to be a polymorphic locus. The entire length of the genomic DNA insert in clone 11H was only 86 bp. It did contain the STR $(AGC)_{12}$, but it was adjacent to the MCS. Clone 28H encoded the STR $(TGC)_8$, which was considered

too short. Clone 36H encoded the STR $(AGC)_5$, which was considered too short. Clone 14H encoded the STR $(AGC)_{14}$, but it ended at a *Hin*P1I site which may have ligated to a non-contiguous fragment from the Spoonbill genome. This locus was tested for polymorphism by designing the primers 14A and 14B:

14HA: 5'-CGGGAAAGTTAGTGCGAGAG-3'

14HB: 5'-AGGAGGCACTAGGAGGCAAT-3'

The amplification of clone 14H with these primers should have produced a product of 146 bp. A distinct band of about 140 bp was produced from 6 Spoonbill genomic DNA samples which were amplified with these primers at the 62°C annealing temperature. Each of the samples appeared to have produced the same band, therefore, this locus was considered to be non-polymorphic.

Clone 23H was identical to clone 24H. Two STR regions were present within these clones. The STR region $(AGG)_7$ was adjacent to the MCS. However, another region, $(AGC)_{12}(AGG)_{10}$, was present near the center of the cloned DNA. The 23H DNA sequence was compared to the GenBank database by the use of BLASTN 2.2.1 (Altschul *et al*., 1997). The results of the GenBank search showed the nucleotide region 185-254 of clone 23H had a 95% homology (69 out of 72 nucleotides) with nucleotides 1-72 (8,737 bp total) of the *Gallus gallus* L-type voltage-gated calcium channel alpha ChCaChA1D mRNA, complete cds (accession #AF027602). Additionally, over 1 dozen highly homologous (95-100%) matches were made with nucleotides 149-169, indicative of a conserved promoter element. The $(AGG)_7$ region of clone 23H was in this region of the clone which was the 5'-leader of the chicken voltage-gated calcium channel DNA

sequence. Oligonucleotide primers 23A and 23C were designed to amplify the interior STR locus. The sequences of the oligonucleotide primers were as follows:

23A: 5'-GTGCGGTAGGAGGCAAGA-3'

23C: 5'-GTGACACGGGATCTCTCCAT-3'

A 197 bp product was expected from the PCR amplification of Spoonbill genomic DNA. PCR amplification reactions were carried out at 55°, 57° and 59°C annealing temperatures. A polyacrylamide gel (7.5%) analysis of the PCR products showed the presence of a single band in 5 different (13, 14, 16, 18 and 21) genomic DNA samples (Fig. 47). The band from each sample migrated at approximately 280 bp, which was higher than the expected 197 bp product. The presence of a single band indicated the locus was non-polymorphic. This result was not unexpected since this region should be conserved because of its proximity to a gene's promoter region.

The next STR isolations began with the same genomic DNA ligated to the S64/S61 adapter. One capture attempt used 50 pmol of STR-3 with 50 pmol STR-6. Another capture attempt used 50 pmol STR-9 and STR-10. Hybridizations of the genomic DNA to the STR oligonucleotides were performed at 37° for 20 minutes, which was the lowest hybridization temperature attempted. The remainder of the STR procedure was followed as directed in Materials and Methods. Eight different agar plates of transformed cells were created: 4 *Hin*P1I 9/10 A, B, C, D, and 4 *Hin*P1I 3/6 A, B, C, D. Each of the plates contained about 100 white colonies and 100 blue colonies. A Colony/Plaque Screen™ membrane was used on each plate to obtain a representative amount from each of the colonies. The membranes were treated to denature and affix the

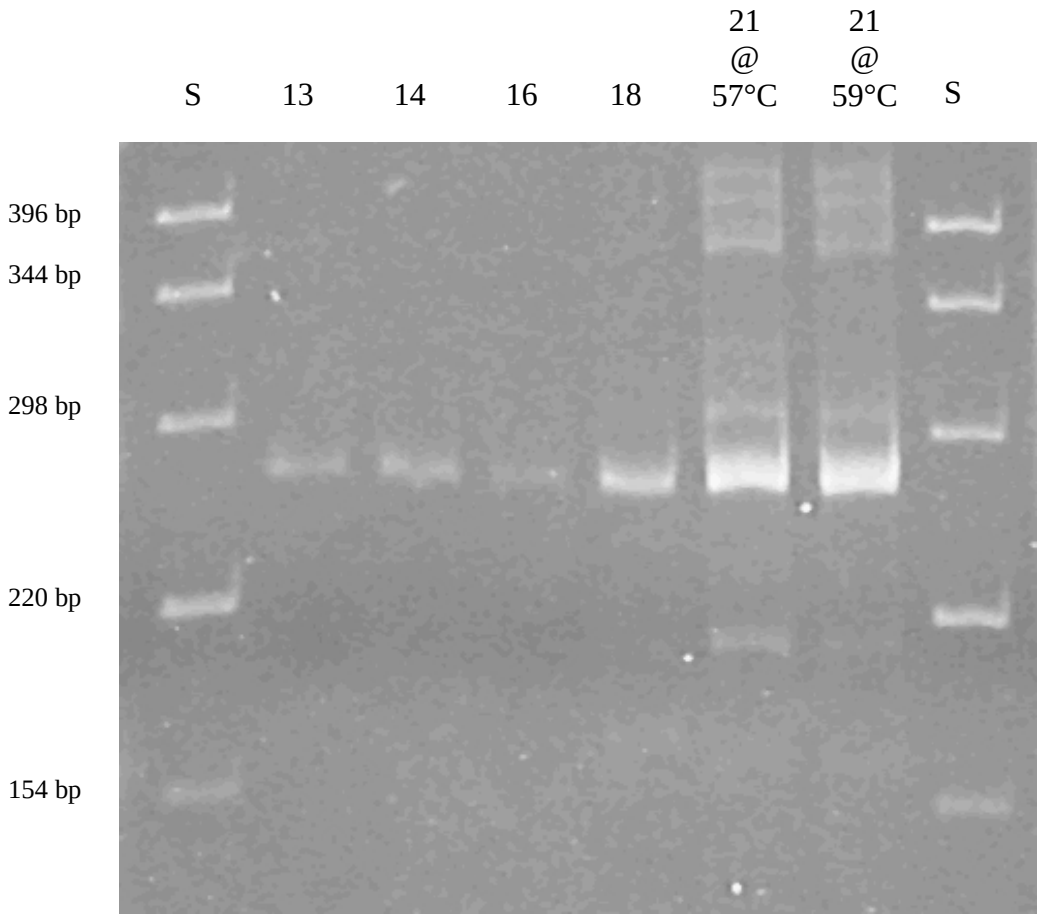

Figure 47. **Electrophoretic analysis of 23A/C amplified Spoonbill genomic DNA.** The Spoonbill genomic DNA samples, which were used with this primer set, are indicated above each well. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. All of the samples were amplified at 57°C, except the last sample, #21, was amplified at 59°C too. The gel was 7.5% (37.5:1) polyacrylamide which was electrophoresed at 250V for 3.25 hours (920 V/hour).

DNA and then hybridized overnight at 55°C. The probe mixture 3/6 was used with the membranes obtained from the 4 *Hin*P1I 3/6 (3/1/01) bacterial plates. The probe mixture 9/10 was used with the membranes obtained from the 4 *Hin*P1I 9/10 (3/1/01) bacterial plates. The membranes were washed at room temperature 2 times in 2.5X SSC, 0.1% SDS. Autoradiography proceeded for 3 days.

The capture with STRs-9 and -10 at 37°C seemed to work very poorly. All 4 of the *Hin*P1I 9/10 plates showed very little binding of the probes, with nothing indicative of a positively-hybridized colony. The capture with STRs-3 and -6 at 37°C worked relatively well. Nine colonies were chosen to have their plasmids isolated and the DNA sequence determined. One of the clones, 20R, did not produce a profile on the autoradiograph. Clone 17R consisted of a $(GAT)_{12}$ insert into the MCS, with no flanking regions present. Clone 19R encoded the STR $(AGC)_8$ in close proximity to the MCS. Primers for PCR amplification were not designed for 17R or 19R. Clone 21R encoded a short (121 bp) DNA insert with the STR $(TGC)_5(AGC)_4$, which was adjacent to the MCS, and therefore not useful. The 3 clones 26R, 27R, and 28R encoded only a 12 bp insert into the MCS. Clone 25R did not contain a STR region.

Clone 18R did contain a long STR region: $(GAT)_{29}$. However, the DNA sequence was contaminated with another DNA clone. The bacterial colony which was identified as 18R was streaked onto an LB Amp^{50} agar plate to create isolated colonies. Two colonies of different morphology were chosen and their plasmids were isolated. The DNA sequence from each of these clones was determined. The sequence with $(GAT)_{29}$ was identified as clone 18b. The size of the DNA insert was 310 bp (Fig. 48). Oligonucleotides 18A and 18B were designed to PCR amplify this locus in order to

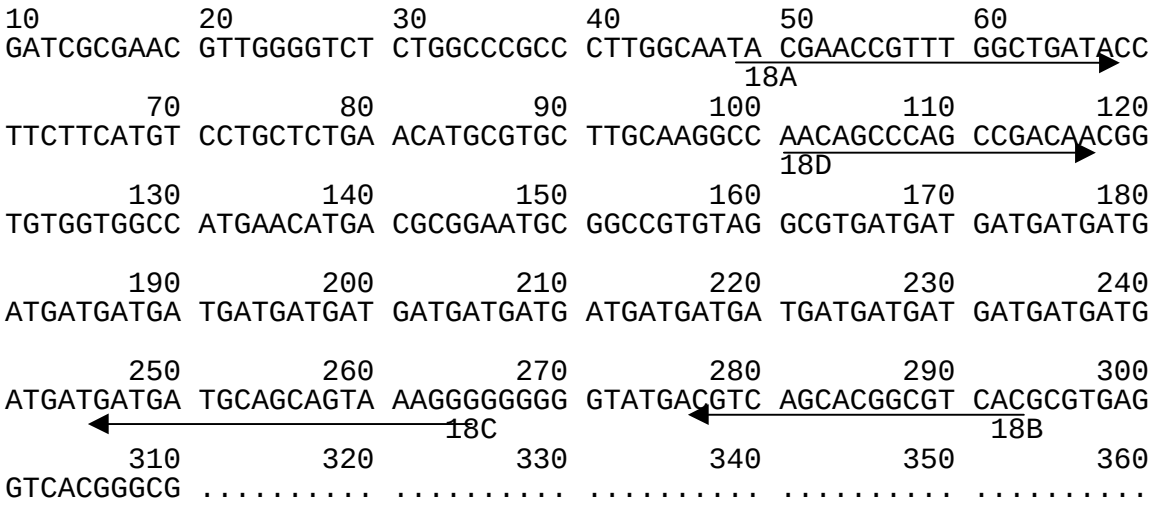

Figure 48. **Nucleotide sequence of STR clone 18b.** The oligonucleotide primers 18A, 18B, 18C, and 18D, and their relative directions, are indicated by the arrows.

create a 255 bp product. PCR amplification of Spoonbill genomic DNA at 60°C annealing temperature resulted in a 700 bp band and primer dimers (approximately 50 bp). When the annealing temperature was dropped to 57°C, 700 bp and 500 bp bands were produced. PCR amplifications were then performed at 55°C annealing temperature while using 3 different concentrations of $MgCl_2$. The 500 bp bands were still present with the normal 1.5 mM $MgCl_2$, whereas the use of 2 mM and 2.5 mM $MgCl_2$ in the PCR reactions only caused the 500 bp to become less distinct as the concentration was increased.

A new primer, 18C, was designed to be 20 bases downstream of 18B. PCR amplifications with the 18A/C primer set on Spoonbill genomic DNA at multiple annealing temperatures (52, 55, 57 and 62°C) resulted in a single band at about 50 bp. A new primer, 18D, was designed to bind downstream of 18A and PCR amplify a 165 bp product with 18C. The PCR amplification of 18B with 18D was expected to produce a 194 bp product. The DNA sequences of the oligonucleotide primers are:

18A: 5'-ACGAACCGTTTGGCTGATAC-3'

18B: 5'-CGTGACGCCGTGCTGAC-3'

18C: 5'-CCCTTTACTGCTGCATCATC-3'

18D: 5'-AACAGCCCAGCCGACAAC-3'

The amplification of Spoonbill genomic DNA samples with 18C and 18D at 59, 60 or 61°C produced 2 bands, one at about 450bp and the other at about 600 bp. The products did not look polymorphic and they were too large relative to the expected size. PCR amplification of Spoonbill genomic DNA with the primers 18B and 18D at the 58°C annealing temperature resulted in the production of multiple bands which were visible on

a 7.5% polyacrylamide gel (Fig. 49). Two bands were produced at about 550 bp. A single band was produced at about 200 bp. Another single band was produced at about 150 bp. Multiple bands were produced from about 390 to 490 bp. A comparison of 4 different Spoonbill samples at this region revealed a polymorphic pattern. However, the products of PCR amplification with 18B and 18D were much larger than expected. The use of these primers to differentiate between individual Spoonbill samples in the range of 390 to 490 bp may be of limited value. The locus may be further characterized by the isolation of clones from this region, which was not performed in the course of this study.

Two more attempts to isolate STR loci were initiated during the sequencing and analysis of clone 18b. The *Hin*P1I/S64/S61 adapter DNA was used for the following capture techniques. One isolation attempt used STR-4 (100 pmol) at the 55°C hybridization temperature. The other isolation attempt used STRs-9 and -10 (50 pmol each) at the 55°C hybridization temperature. The procedure was followed as outlined in Materials and Methods. Eight agar plates of transformed cells were created: 4 *Hin*P1I STR-4 A, B, C, D, and 4 *Hin*P1I STRs9/10 A, B, C, D. Each of the plates contained about 40 white colonies and 120 blue colonies. In order to better select positively-hybridized colonies, 4 master plates, designated with an "A", and duplicates of each designated with a "B", were created from all of the white colonies on these plates. A Colony/Plaque Screen™ membrane was used on each "A" plate. The membranes were treated to denature and affix the DNA and then hybridized overnight at 55°C. The probe mixture PSTR-4 was used with the membranes obtained from the 4 *Hin*P1I STR-4 (3/6/01) bacterial plates. The probe mixture 9/10 was used with the membranes obtained

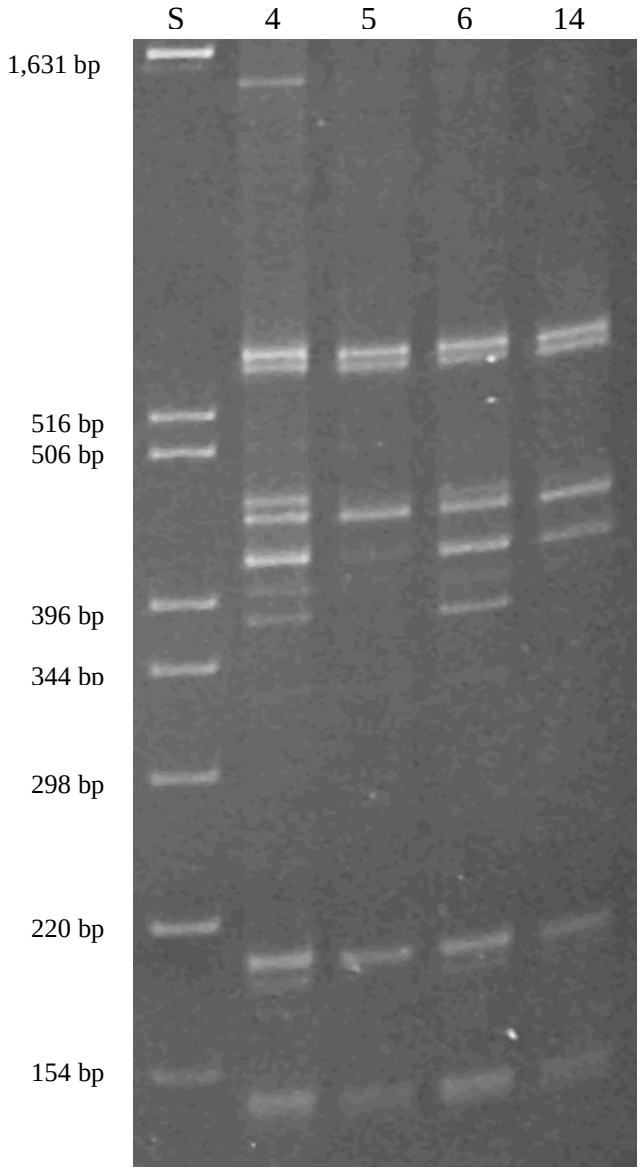

Figure 49. **Electrophoretic analysis of 18B/D amplified Spoonbill genomic DNA.** The Spoonbill genomic DNA samples were amplified at 58°C annealing temperature and are indicated above each well. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. The gel was 7.5% (37.5:1) polyacrylamide which was electrophoresed at 100V for 10 hours.

from the 4 *Hin*P1I STR-9/10 (3/6/01) bacterial plates. The membranes were washed 2 times at room temperature in 2.5X SSC, 0.1% SDS. Autoradiography proceeded for 12 hours.

None of the approximately 200 master plate streaks of STR-4 captured DNA clones were positively-hybridized. Four of the approximately 180 master plate streaks of STR-9/10 captured DNA clones were indicated as positively-hybridized. The DNA sequences of these 4 clones were determined. Clone 3/4F appeared to have no STR insert. Clones 3/6F and 3/13F were identical and encoded the STR $(GT)_9$. The size of the DNA region containing the STR was effectively 89 bp since a *Hin*P1I restriction endonuclease site was near the center of the clone. The short (9) repeated motif of the STR, and the overall size of the clone made this locus undesirable for primer design.

Clone 3/24F encoded the STR motif $(CT)_3(GT)_{21}$ (Fig. 50). The total size of the DNA insert was 197 bp and is listed in GenBank as accession #AF469479 (Fig. 51). The primers 24A and 24B were designed as follows to amplify a product of 169 bp:

24A: 5'-AGCAACTGGAGAAGGACCAG-3'

24B: 5'-CCCGTTCCAGATGCTGCTAT-3'

An amplification at the 58°C annealing temperature created products which were approximately the correct size (about 150 bp), but were not distinct (Fig. 52). Attempts to amplify genomic DNA at 55°C seemed to produce the lowest band in only some of the samples (data not shown). The $MgCl_2$ concentration was adjusted in the PCR amplifications to 2 mM and 2.5 mM at 55°C annealing temperature, which yielded slightly more distinct areas of product at 2 mM. When 12 samples were amplified at 60°C, and 1.5 mM $MgCl_2$, a slightly different pattern was observed (Fig. 53).

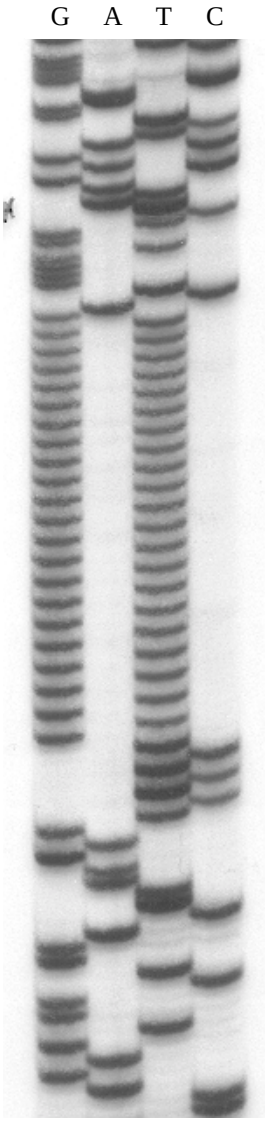

Figure 50. **Autoradiograph of the DNA sequencing gel with the STR clone 3/24F.** This STR region, locus Aajμ6, and its immediate flanking sequences have the GenBank accession #AF469479.

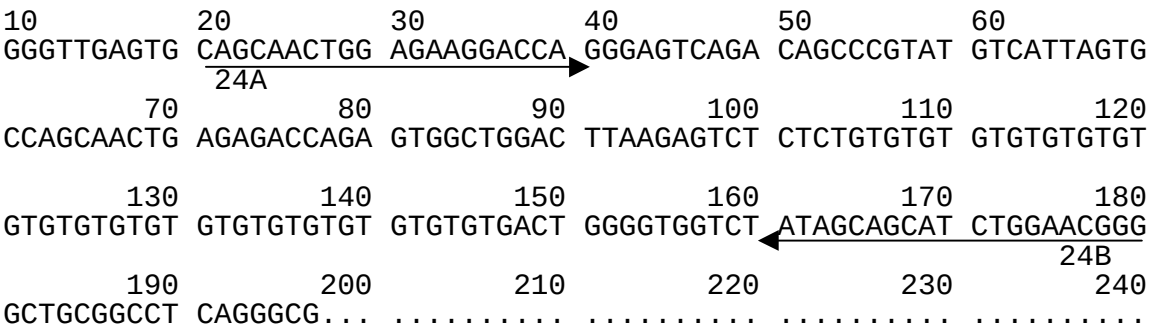

Figure 51. **Nucleotide sequence of STR locus Aajμ 6 (3/24F).** The oligonucleotide primers 24A and 24B and their relative directions are indicated by the arrows. This DNA sequence has the GenBank accession #AF469479.

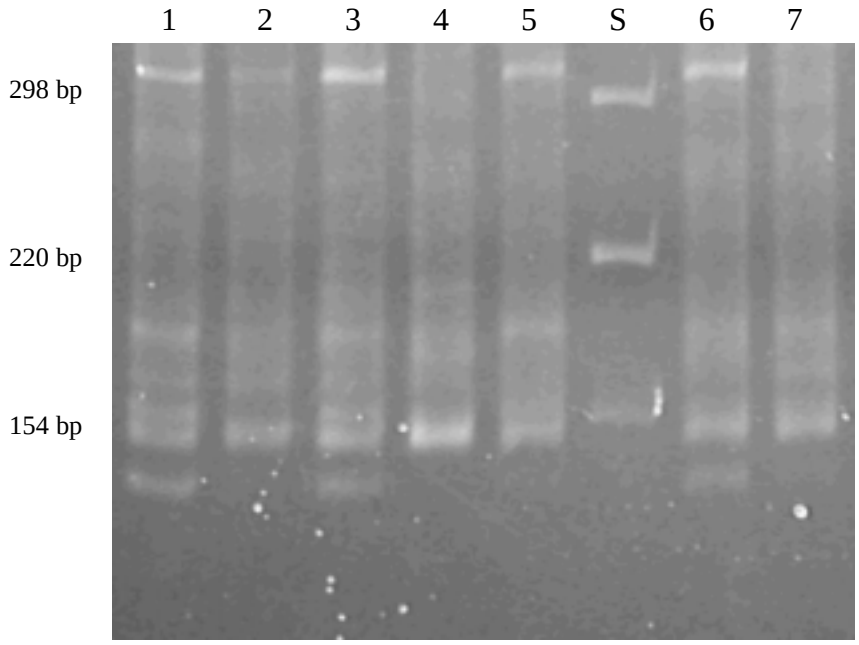

Figure 52. **Electrophoretic analysis of 24A/B amplified Spoonbill genomic DNA at 58°C.** The Spoonbill genomic DNA samples are indicated above each well. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. The gel was 7.5% (37.5:1) polyacrylamide which was electrophoresed at 200V for 5 hours.

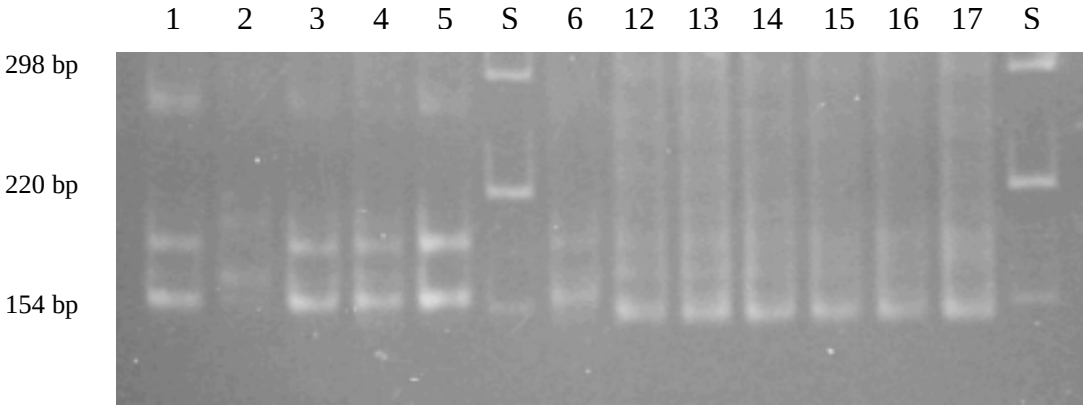

Figure 53. **Electrophoretic analysis of 24A/B amplified Spoonbill genomic DNA at 60°C.** The Spoonbill genomic DNA samples are indicated above each well. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. The gel was 7.5% (37.5:1) polyacrylamide which was electrophoresed at 70V for 15 hours.

More attempts were made to optimize the results of PCR amplifications at this locus. One amplification was programmed to adjust the annealing temperature up by 1° every cycle from 55°C to 63°C (then held at 63°C for the remaining cycles), with no improvement in the results. Another amplification was performed at 62°C. Some samples yielded products with more distinct bands, whereas other samples yielded no products. The next amplification used a change in the thermal cycle parameters. The parameters were designed to "step-down" from 68°C to 58°C, or 65°C to 60°C, in 1° increments every cycle until the lower temperature was attained and held for the remaining cycles. Additional primers (24A2, 24C, 24D and 24E) were designed to increase the melting temperature of the original primers. PCR amplifications with these primers did little or nothing to produce better products. The difficulties of amplifying a specific DNA product from each sample was most likely a result of the dinucleotide nature, $(GT)_{21}$, of the STR locus. The phenomenon of *Taq* DNA polymerase slippage during DNA replication is more pronounced through a dinucleotide STR than a tri- or tetranucleotide STR locus (Robert Benjamin, personal communication).

The next attempt to capture STR loci began by using just STR-3 (100 pmol) as the capturing oligonucleotide. The hybridization to capture STR loci was performed at 55°C and lasted 20 minutes. The last 4 washes of the beads with the captured DNA were performed at 55°C. Duplicate master plates, STR-3 (3/13/01) A1 and A2, were created from the 4 plates of transformed cells. A Colony/Plaque Screen™ membrane was used to create a bacterial image of plate A1, and then it was treated to denature and affix the DNA. The STR probe 3/6 was used in an overnight hybridization at 55°C. After 2 washes at room temperature (23°C) the membrane was exposed to X-ray film for 20

hours. Twelve positively-hybridized areas were chosen for plasmid DNA isolation and DNA sequence determination.

Two of the clones (3/32 and 3/48) did not produce a DNA sequence profile on the autoradiograph. Seven of the clones (3/21, 3/25, 3/28, 3/29, 3/36, 3/41, 3/43) encoded the expected STR insert $(GAT)_n$, with "n" ranging from 10 to over 100, but each of the STR loci were immediately flanked by the MCS. Three of the clones (3/31, 3/46, 3/47) had STR loci with a sufficient amount of flanking DNA to design PCR primers. The characteristics of each STR clone are listed in Table IX.

Table IX. **Analysis of STR clones from 3/13/01**

Clone ID	DNA sequence	Notes	Amplification primers designed?
3/21	(GAT) x 100+	STR at MCS	
3/25	(GAT) x 63	STR at MCS	
3/28	(GAT) x 10	STR at MCS	
3/29	(GAT) x 10	STR at MCS	
3/31	$(ATC)_8AAT(GAT)_9$		Primers designed
3/36	(GAT) x 14	STR at MCS, same as #41	
3/41	(GAT) x 14	STR at MCS, same as #36	
3/43	(GAT) x 12	STR at MCS	
3/46	(GAT) x 15		Primers designed
3/47	(GAT) x 29		Primers designed

Problem: Primers cannot be designed when the STR is at the MCS.

Primers 31A and 31B were designed to amplify the STR locus of clone 3/31. The expected size of this amplified region was 362 bp. An additional primer, 31C, was created to reduce the expected size of the 31A/C PCR product to 232 bp. The nucleotide sequences of the primers were as follows:

31A: 5’-AGCAGGCCCAAATAGTTCAC-3’

31B: 5’-CACCCTGTGTCACCCACTG-3’

31C: 5’-CCACCAACATCACCATCATC-3’

The amplification of 6 Spoonbill genomic DNA samples with the primer set 31A/B at 56°C resulted in products of about 600 bp, 140 bp, and 80 bp (data not shown). Attempts to amplify the same samples at 55°, 60°, and 62°C, along with adjustments of the $MgCl_2$ levels (2, 2.5 and 3 mM) resulted in the production of the 600 bp band only. Amplification of Spoonbill DNA with the primer set 31A/C at the 55°C annealing temperature resulted in no products. It is unclear as to why the expected products cannot be amplified from this locus. One explanation is that the palindromic nature of the STR, $(ATC)_8AAT(GAT)_9$, interfered with the amplifications by the hybridization to itself. The amplifications at this locus were considered to be non-polymorphic.

Primer sets were designed to amplify the STR loci of 3/46 and 3/47, with the consideration that they encoded an identical flanking sequence. The STR loci differ in the number of repeats (one has 15 repeats, the other has 29) and the remaining flanking sequence of each clone, which appear to be unique. The following primers were designed to amplify these loci:

46A: 5’-CAGCCCAGTGTTGGCTTACT-3’

46B: 5’-CAGCCAAGTAGGCAGAGTCA-3’

46C: 5’-CTGAGGGAAGGGTTCACAAT-3’

47A: 5’-TCCAGCTACAACCTTTCCTGA-3’

The expected product size of 46A/B was 186 bp. The expected product size of 47A and 46B was 195 bp. The complementary sequence to 46C was designed to be 30

bases closer to the STR. PCR amplification of Spoonbill genomic DNA at 60°C with primer set 46A/B resulted in multiple bands visible on a 7.5% polyacrylamide gel. Each sample contained the same bands at about 90, 100, 180, 190, and 240 bp. The locus did not appear to be polymorphic. PCR amplification of Spoonbill genomic DNA at 55°C with primer set 47A/46B resulted in no products. Attempts to amplify each locus at 62° annealing temperature resulted in no products. However, amplification at 59°C with the primers 46A and 46C produced a distinct band at about 450 bp, which was larger than the expected product size. PCR amplification at 59°C with the primers 47A and 46C produced no products. Neither primer set resulted in the amplification of a polymorphic locus. They were both considered to be non-polymorphic.

The next 2 STR capture attempts were performed with STRs -6 and -8 (75 pmol each) and STRs -9 and -10 (75 pmol each). The DNA fragments (size-selected and ligated to the adapters) were hybridized to the STR oligonucleotides at 55°C for 20 minutes. The last 4 washes were performed at 55°C to help select against any clones which were not at least 8 repeats long. The DNA from each capture procedure was eluted by the addition of 20 µl 2X PCR amplification buffer, followed by a 10 minute incubation at 95°C. The solutions were immediately removed from the beads (12 µl 6/8 captured DNA and 14 µl 9/10 captured DNA) and subjected to amplification reactions with oligonucleotide S61. The amplified DNA was processed as outlined in Materials and Methods. The subsequent transformations yielded slightly smaller colonies than normal and only about 20 white colonies and 40 blue colonies per plate.

Master plates SB 6/8 repA1 (3/15/01), SB 6/8 repB1 (3/19/01), SB 9/10 repA1 (3/19/01) were created from these transformants. The colony lift membranes were

hybridized overnight with their corresponding probes at 55°C. After autoradiography for 5 hours, only 2 bacterial streaks on the master plate SB9/10 repA1 appeared to be strongly hybridized. No positive light-ups were visible on the SB 6/8 master plate membranes. The plasmids from the 2 hybridized bacterial clones (3/40 and 3/41) were isolated and the DNA sequences were determined. Both clones possessed relatively short STR regions. Clone 3/40 encoded the STR motif $(GT)_{20}$. Clone 3/41 encoded the STR motif $(GT)_{15}$. However, clone 3/41 was contaminated with another plasmid making it impossible to design primers which were specific to the STR plasmid. Attempts were made to purify the STR clone 3/41 with no success. Primers 40A and 40B were designed to amplify clone 3/40. Their DNA sequences are as follows:

40A: 5'-GGTATCGCGTGTGCAAGG-3'

40B: 5'-GCCACCCCGTGCTTTAC-3'

PCR amplification of Spoonbill genomic DNA with primers 40A and 40B was expected to yield a 109 bp product. PCR amplifications at 56, 58 and 60°C each resulted in bands of about 50 bp. Additionally, since each of the Spoonbill samples resulted in the same band, this locus was considered to be non-polymorphic.

The next attempts to capture STR loci used a mixture of STRs-3 and -6 (100 pmol each) or STRs-6 and -9 (75 pmol each). Four separate capture experiments were performed on Spoonbill genomic DNA samples. Five micrograms of DNA (1 µg from 5 different birds) was digested with either *Sau*3AI or *Hin*P1I and size selected to be 300-800 bp. Each of the STR pairs (3/6 or 3/9) was used to capture STR loci from each type of DNA digestion. The hybridizations of STR oligonucleotides to digested DNA samples were allowed to proceed for 2.5 hours at 55°C (for *Sau*3AI) or 40 minutes at 55°C (for *Hin*P1I). The last 4 washes for all capture experiments were performed at 55°C and the DNA was eluted with fresh 0.15 M NaOH as outlined in Materials and Methods. Only 5 µl, out of the 30 µl recovered from each capture technique, was used for amplifications with oligonucleotide S61. A 5 µl portion of each 50 µl PCR product was electrophoresed on a 2% agarose gel to verify the presence of amplified DNA. Three out of 4 samples produced the expected smear: *Hin*P1I 3/6, *Sau*3AI 3/6, and *Hin*P1I 6/9. The remainder of each product (45 µl) was ethanol precipitated and digested as before. One half (10 µl) of the ligation reactions were used in transformation experiments. Each agar plate (8 total) produced about 35-45 white colonies and 30-40 blue colonies.

Three sets of duplicate master plates were produced: Master 3/6 6/3/01A & B, Master 3/6 6/10/01 A & B, and Master 6/9 6/10/01 A & B. Separate Colony/Plaque Screen™ membranes were used to lift the colony streaks from each "A" plate. These membranes were treated to denature and affix the DNA and were then hybridized overnight at 55°C with the appropriate probes. The membranes were washed 2 times at room temperature and then exposed to film 9 hours. Nine positively hybridized areas were aligned with their original colony streaks, which were subsequently processed

through a rapid plasmid isolation procedure. Each of the clones (16/2, 16/23, 16/30, 16/32A, 16/33, 16/34, 16/38, 16/40, 16/41) was processed through the DNA sequencing procedure. The characteristics of each locus are listed in Table X.

Table X. **Analysis of STR clone set 16A**			
			Amplification
Clone ID	DNA sequence	Notes	primers designed?
16/2	STR absent	No STR visible	
16/23	(AGC) x 8		
16/30	(AGC) x 9	STR at MCS and only 42 bp insert	
16/32	(GT) x 15	STR at MCS	
16/33	(AGC) x 9	STR at MCS	
16/34	(GAT) x 10	One flank same as #40, truncated flank	
16/38	(GT) x 38		Primers designed
16/40	(GAT) x 11	One flank same as #34: GAT degenerates	
16/41		No sequence obtained	
Problem: Primers cannot be designed when the STR is at the MCS			

Four of the clones (16/30, 16/32, 16/33, 16/41) failed to produce a DNA sequence profile. Each of these was processed through the plasmid isolation procedure again. Three of these samples (16/23, 16/34,16/40) produced a DNA sequence profile, whereas clone 16/41 did not. Seven of the clones carried an STR locus. The STR locus for clone 16/38 looked exceptionally promising since motif was (GT) with 38 repeats. The primers needed for the PCR amplification of clone 16/38 were designed as follows:

38A: 5’-CCCAGGCAAAGAAACAAACA-3’

38B: 5’-TCGGGGTATCTCCTGCATAA-3’

PCR amplification with this primer set was expected to produce a band of 160 bp. Spoonbill genomic DNA was amplified at 55, 57, 59, 60 and 62°C. Three distinct bands were produced at approximately 80, 450 and 600 bp, and extremely faint bands of about 150 bp were produced at 57°C in 2 out of 14 samples. This locus was considered to be non-polymorphic. However, it is possible that new primers could be designed for this locus to prove it to be polymorphic.

The most recent set of STR capture techniques (3 different captures) ended with the use of 5 µl, from a total of 30 µl recovered from the elution and cleanup of DNA constructs. The remaining 25 µl from each capture attempt were used to elute more STR loci by processing 5 µl aliquots from each sample through the remainder of the STR capture technique. This allowed the creation of 12 more master plates: 4 were labeled as set 16B, and 8 were labeled as set 16C. A separate Colony/Plaque Screen™ membrane was used on each plate. Each of the membranes was treated to denature and affix the DNA, then were hybridized overnight at 55°C with the appropriate probes. After 2 room temperature (23°C) washes the membranes were exposed to x-ray film for 20 hours.

There were a total of 28 bacterial streaks which were positively hybridized. Each of the bacterial streaks was chosen for a plasmid DNA isolation and subsequently passed through a DNA sequencing protocol. The characteristics of each clone are listed in Table XI. PCR primers were designed for 5 clones. The primers for locus 4B were designed as follows:

4F: 5’-AATTGTTGGCCAGAGCAGAG-3’

4R: 5’-CAGACAGGGCCACCTATTTC-3’

Table XI. **Analysis of STR clone sets 16B and 16C**			
			Amplification
Clone ID	DNA sequence	Notes	primers designed?
16/1B	(CCT)7(GCT)3	STR at restriction site, same as #2B	
16/2B	(CCT)7(GCT)3	STR at restriction site, same as #1B	
16/2C	(AGC) x 7		
16/3B	(AGC)3(AGT)2(AGC)3	degenerated STR	
16/4B	(AGC) x 10		Primers designed
16/5B	(AGC) x 5	STR too short, same as 6B & 7B	
16/6B	(AGC) x 5	STR too short, same as 5B & 7B	
16/7B	(AGC) x 5	STR too short, same as 5B & 6B	
16/8B	(GAT) x 15	STR at MCS	
16/8C	(AGC) x 8		
16/12B	(GAT)3GGT(GAT)13	degenerates into AAT among GAT	Primers designed
16/27C	(AGC)6AGT(AGC)7	STR at MCS	
16/29B	(AGC) x 9	same as #30B	Primers designed
16/30B	(AGC) x 9	same as #29B	
16/30C	no visible STR	no visible STR	
16/32C	(GAT) x 15		Primers designed
16/33B	no visible STR	no visible STR	
16/33C	no visible STR	contaminated sample	
16/34B	(GAT) x 13	STR at restriction site	
16/37C	(AGC) x 5	STR too short	
16/40B	(AGC) x 7	STR at MCS	
16/40C	(AGC) x 7	additional flanking sequence needed	
16/41B	(AGC) x 9	additional flanking sequence needed	
16/43B	(AGC) x 6	STR too short	
16/43C	(AGC) x 5	STR too short	
16/44B	(AGC) x 8	contaminated sample	
16/45B	(AGC) x 14		Primers designed
16/46C	no visible STR	poor quality sequence	
Problem: Primers cannot be designed when the STR is at the MCS.			

The expected product size from the amplification of Spoonbill genomic DNA was 281 bp. When amplifications were attempted with the primers at the 60°C annealing temperature, only a very faint band appeared at about 300 bp on a 2% agarose gel (80 V, 1.5 hours). Amplifications at 58 and 62°C resulted in only smears visible on the gels. At the 55°C annealing temperature, multiple bands were formed from 400 bp to 100 bp. Two more amplification reactions were performed at 51 and 54°C, with each of these resulting in very faint bands about 300 and 500 bp. No indication of polymorphism was detected. Future studies could be performed on this locus to ensure its non-polymorphic status.

Two primers were designed to amplify the locus 16/12B:

16/12A: 5'-CCGGCAACTAAGCACCATAG-3'

16/12B: 5'-CAGGTCTGTCAGTCTCTGAGTCC-3'

The expected product size resulting from amplification of Spoonbill genomic DNA was 275 bp. Amplification at the 58°C annealing temperature yielded no results. Currently, amplifications at this locus are considered to be inconclusive.

A set of primers was designed for the clone 16/29B. The primers were:

29A: 5'-TAGGCTCTTGCCCAGACTGA-3'

29B: 5'-CTGATTAAGAGCACGCATGG-3'

The expected size of the amplified product was 197 bp. Amplifications were performed at the 57° annealing temperature which resulted in a product of about 80 bp. Additional amplifications were performed at the 57°C annealing temperature which resulted in bands at 80, 150 and 280 bp. Amplification at the 60°C annealing temperature resulted in the

same 3 products, except the 80 and 280 bp products were more distinct. This locus was considered to be non-polymorphic.

A set of oligonucleotide primers was designed for the clone 16/32C as follows:

32A: 5'-CCCTCCCAAGTGGTTACAGA-3'

32B: 5'-CTTGCATGTTCCAAATGGTG-3'

The expected size of the amplified product was 200 bp (Fig. 54). An amplification at the 58°C annealing temperature with 6 Spoonbill genomic DNA samples (#1-#6) resulted in products of approximately 200 bp (2% agarose gel; 80V, 1.5 hours). Additional samples (#7-#13) were amplified as before and then all of the samples were loaded on a 7.5% (37.5:1) polyacrylamide gel and electrophoresed at 150 V for 6.5 hours (Fig. 55).

Two distinct bands were visible on the polyacrylamide gel. A single band was present at about 200 bp from every sample which was amplified. A visual correlation of the known sex types of each individual Spoonbill (Tables IV, V and VI) with the banding patterns observed on the polyacramide gel confirmed a suspicion that the patterns were sex specific. The upper band was amplified from the Z chromosome and the lower band was amplified from the W chromosome.

Spoonbill samples #23 through #42 were then amplified with one of the oligonucleotide primers end-labeled with γ-^{32}P dATP. One-tenth (2.5 µl) of each PCR reaction (25 µl) was diluted with 2 µl Sequenase™ stop solution (formamide-based gel loading solution). All of the samples were then heat denatured (95°C) for 2 minutes and immediately placed on ice. The entire set of samples was then loaded on a 6% denaturing sequencing gel next to a clone from which the DNA sequence had been

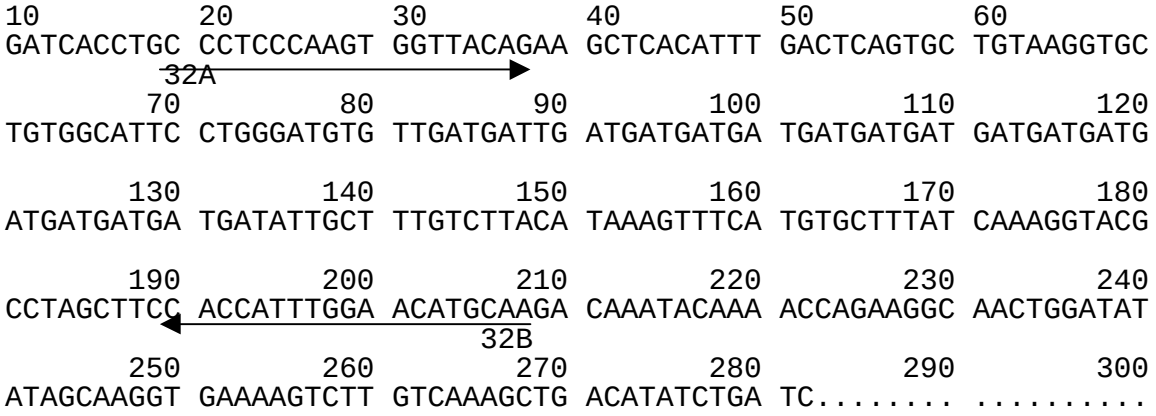

Figure 54. **Nucleotide sequence of STR locus Aajμ4.** The primers 32A and 32B and their relative directions are indicated by the arrows. This sequence has the GenBank accession #AF469477.

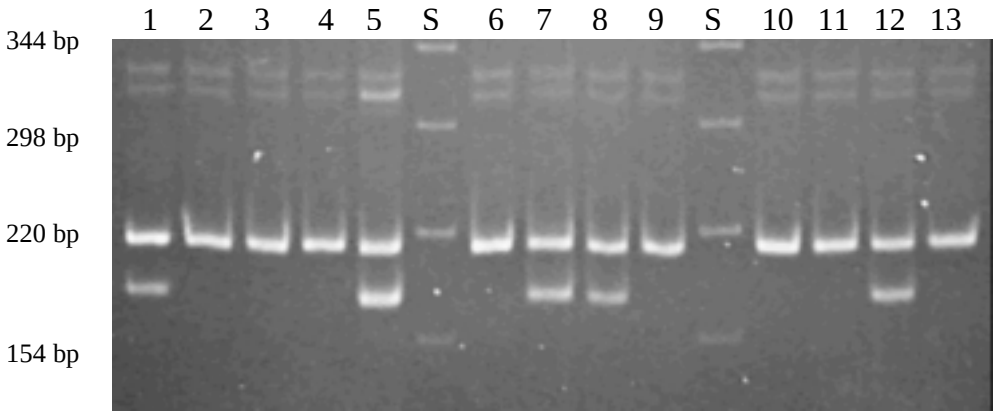

Figure 55. **Electrophoretic analysis of 32A/B amplified Spoonbill genomic DNA.** The 13 Spoonbill genomic DNA samples used with this primer set are indicated above each well. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. This 7.5% (37.5:1) polyacrylamide gel was electrophoresed at 150V for 6.5 hours.

determined with α-^{35}S dATP. The gel was electrophoresed at 50 W for 3 hours and was then dried and prepared for autoradiography as described in Materials and Methods. An autoradiograph of the banding pattern allowed for an estimation of the band sizes. The upper band was at the expected 200 bp and the lower band was at 161 bp. The entire collection of Roseate Spoonbills (51 samples) was PCR amplified with the primer set 32A/B. The sex of every bird was confirmed or established, which validated the utility of this test. All future studies of the Roseate Spoonbill can now use this PCR-based test to determine sex.

PCR primers were designed for locus 16/45B as follows:

45BF: 5'-GGGGGCAGCTTGTCCAT-3'

45BR: 5'-CCTGCCCTTCTGATGCAGAGC-3'

The expected product size from amplification with this primer set is 241 bp. When Spoonbill genomic DNA was amplified at the 60°C annealing temperature it appeared to produce 2 bands (200 and 1000 bp) in a 2% agarose gel (80 V, 1.5 hours). The annealing temperature was raised to 63°C and the amplified products still appeared to be the same on a 2% agarose gel (80 V, 1.5 hours). A portion (8 µl) of each sample was then loaded on a 7.5% (37.5:1) polyacrylamide gel and electrophoresed at 140 V for 7 hours (Fig. 56). Two distinct bands were present on the gel; one at about 240 bp and the other at about 1000 bp. Neither banding pattern appeared to be polymorphic. Therefore, locus 16/45B was considered to be non-polymorphic.

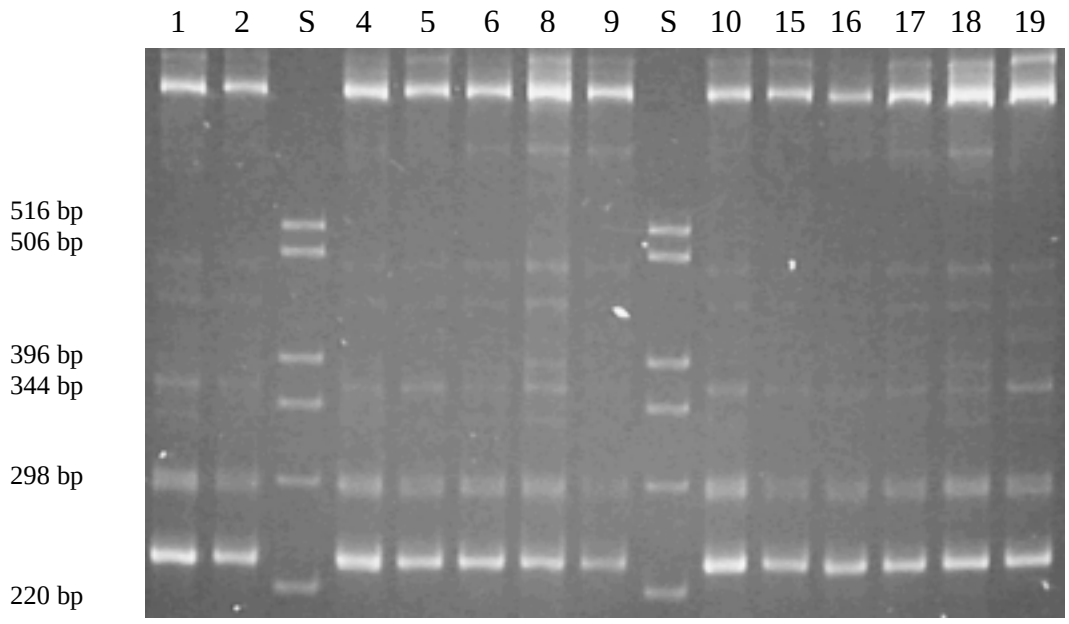

Figure 56. **Electrophoretic analysis of 45BF/R amplified Spoonbill genomic DNA at 63°C.** The 13 Spoonbill genomic DNA samples amplified with this primer set are indicated above each well. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. This 7.5% (37.5:1) polyacrylamide gel was electrophoresed at 140V for 7 hours.

STR isolations from lambda ZAP Express® libraries

The isolations and subsequent characterizations of microsatellite loci by the magnetics-based procedure proved to be very labor intensive. Most importantly, much less than 1% of the clones, which were captured and hybridized with the biotin-labeled STR oligonucleotides, yielded a polymorphic locus. An average STR capture attempt yielded only 10 to 50 transformed (white) colonies (10). Some of the procedures would yield hundreds of transformed colonies. Well over 2,000 colonies were transferred either to master plates or lifted directly from their transformation plates by the Colony/Plaque Screen™ membranes, then subsequently hybridized. While repeated cycles of this technique did eventually yield 4 characterized polymorphic STR loci, an alternative technique was employed to more quickly and efficiently isolate STR loci. The new approach relied upon the creation of small-insert (400-800 bp) Spoonbill genomic DNA libraries within a phagemid vector which, although not enriched for STR loci, could be plated out at high density (10,000 to 30,000 pfu/plate) and screened for clones containing microsatellite regions using synthetic tri- and tetranucleotide repeat probes.

Two Spoonbill genomic DNA libraries (*Sau*3AI/*Bam*HI and *Tsp*509I/*Eco*RI) were constructed in the lambda ZAP Express® phagemid vector. Each lambda ZAP Express® library was titered and tested for background (ZAP Express® vector with no inserts) as described in Materials and Methods. The recombinant lambda ZAP Express® clones appeared as 1 mm zones of clearing (colorless plaques) on a bacterial lawn. The non-recombinant plaques were identified by their blue color. The titer of the *Tsp*509I/*Eco*RI library was 3×10^5 pfu/ml with approximately 0.1% blue plaques. The titer of the *Sau*3AI/*Bam*HI library was 3×10^5 pfu/ml with about 0.3% blue plaques.

Two 82 mm phage plates, one with 10,000 pfu and the other with 30,000 pfu (confluent lysis), were created from each ZAP Express® library for the initial screening for the presence of STR loci. Replica lifts (A and B) were performed on each phage plate (4 plates for a total of 8 lifts) with the Colony/Plaque Screen™ membranes. A DNA probe was used to identify any clones within the libraries which may contain a specific STR. An analysis of the DNA sequences at loci obtained via the previous STR capture attempts showed a greater proportion of the $(GAT)_n$ STR locus compared to any other STR locus. A probe made from an STR clone with a large number of repeats would be superior both because of its ability to be more highly labeled and the increased stability and thus specificity of its hybridization with other target loci.

The production of such a probe began with the STR clone 3/21, which encoded the STR locus $(GAT)_{100+}$ (Table IX). The forward (-40) and reverse (M13R) sequencing primers were used to PCR amplify this segment from 2 ng of the 3/21 plasmid while incorporating the radiolabeled nucleotide α-^{32}P dATP. The creation of a PCR amplified probe began with the following mixture:

2.5 µl	rapidly isolated plasmid DNA (approximately 2 ng)
5 µl	10X PCR Buffer A, includes 15 mM $MgCl_2$ (FisherBiotech™)
4 µl	F (-40) primer (5 µM)
4 µl	R (M13) primer (5 µM)
3 µl	d(GTC) mix: 2.5 mM each dNTP
5 µl	α-^{32}P dATP
0.1 µl	*Taq* DNA polymerase (5 U/µl) (FisherBiotech™)
26.4 µl	ddH_2O

50 µl Total volume

The DNA Thermal Cycler (Perkin-Elmer Cetus Model No. N801-0180) parameters were programmed to begin with 3 minutes at 94°C. This was followed by 30 cylces of 94°C for 1 minute, 55°C for 45 seconds, and 72°C for 1 minute. The temperature was then held at 72° C for 5 minutes followed by a constant hold at 4°C. The amplified probe was purified from the unincorporated nucleotides by passing the sample through a Sephadex™ G-25 (Pharmacia Biotech) spun column (see Materials and Methods). Approximately 50 µl of the probe, at 50,000 cpm/µl, was recovered.

The amplified probe was tested for problems by hybridizing it with lifts from 2 plates of large-insert lambda phage clones. The Lambda DASH® II Spoonbill genomic library, which was created in 1997, was plated out to create two plates; one of 10,000 pfu and the other of 30,000 pfu. Four lifts, using the Colony/Plaque Screen™ membranes, were created from the 2 titered plates (A and B lifts each) of lambda phage clones. After treatment to denature and affix the DNA, the 4 lifts were hybridized with 1 million cpm (20 µl) of the denatured (2.5 minutes at 100°C) probe. The hybridization proceeded for 18 hours at 65°C and was concluded by the washing of the membranes twice at room temperature with 2.5X SSC, 0.1% SDS. After the washes, the membranes still bound in excess of 500 cpm, which was consistent with the probe binding to some areas (plaques) very well, or alternatively, non-specifically to large areas of the membranes. Therefore, the membranes were washed at 65°C for 20 minutes with 2.5X SSC, 0.1% SDS, and were subsequently determined to have approximately 300 cpm of bound probe. An autoradiograph of the membranes (20 hours) indicated a very high level of non-specific probe binding. Only 2 positively hybridized areas appeared on both A and B lifts, which

was much less than expected 20-24 positively hybridized areas per 10,000 pfu. It appeared that the probe made from the clone 3/21 bound to too much of the membrane non-specifically, but also may have bound to plaques containing STR regions.

The same probe was then used in a hybridization experiment with the 8 lifts from the plates created from the lambda ZAP Express® libraries. The lifts were placed in a Techne™ hybridization tube with 15 ml Hybridization Solution and 1 million cpm (20 µl) of the denatured (2.5 minutes at 100°C) probe. The hybridization proceeded for 20 hours at 55°C and was concluded by the washing of the membranes twice at room temperature with 2.5X SSC, 0.1% SDS. After the washes, the membranes still bound in excess of 500 cpm. Therefore, the membranes were washed at 55°C for 20 minutes with 2.5X SSC, 0.1% SDS, and were subsequently determined to possess approximately 300 cpm. An autoradiograph using the high-speed film, Kodak BioMax MR™, for 16 hours showed that the membranes had a non-specific pattern of bound probe. The 8 membranes were then exposed to x-ray film for 72 hours. The non-specific pattern of bound probe was still easily visible. Five positively hybridized regions (all from the same plate: *Eco*RI [B], A and B lifts) could also be identified as possible STR-containing clones. However, the apparent cluster of clones on one plate, and the less than expected 2 positive "light-ups" per 10,000 pfu (Longmire *et al*., 1999), were reasons to search for a better STR-specific probe.

Since the probe 3/21 was not as useful as hoped, a new probe was created by the use of terminal deoxytransferase (TdT) (see Materials and Methods) and the oligonucleotide STR-3 [5' biotin-$(GAT)_9$]. The 8 membranes which had been previously hybridized with the probe 3/21 were stripped of the probe (see Materials and Methods) to

prepare them for hybridization with the new probe. The new probe, STR-3/α-^{32}P, was prepared (see Materials and Methods) and cleaned of excess nucleotides by the use of a G-25 spun column. A total volume of 48 µl was recovered with approximately 250,000 cpm/µl. The 8 stripped membranes were hybridized for 12 hours at 55°C in 15 ml Hybridization Solution and 2.5 million cpm (10 µl) probe. They were washed at room temperature twice with 2.5X SSC, 0.1% SDS. A Geiger counter survey indicated 300-500 cpm per membrane. After autoradiography (48 hours), many of the positive markings were the same as those seen with the $(GAT)_{100}$ probe, except some were more distinct and the background (non-specific) probe binding was substantially decreased (by approximately 50%).

An attempt was made to identify more STR loci from the same set of library clones using 2 additional probes. The same 8 membranes were then hybridized at 55°C with 2 new 3' end-labeled probes: STR-6 $(GCA)_9$ and STR-8 $(CCT)_8$ and washed at room temperature twice with 2.5X SSC, 0.1% SDS. A Geiger counter survey of each membrane detected approximately 500 cpm. Autoradiography of 48 hours revealed 6 strongly positive plaques and 10 weakly hybridized plaques.

Because of the successes isolating positively hybridized clones from these libraries, 8 more plates of phage (10,000 pfu/plate: 4 from the *Sau*3AI/*Bam*HI library and 4 from the *Tsp*509I/*Eco*RI library) were created. Colony/Plaque Screen™ membranes were used on each of these plates (A and B lifts) and were treated to denature and affix the DNA. All 16 membranes were then hybridized overnight at 53°C with the 3 α-^{32}P end-labeled STRs -3, -6 and -8. The membranes were washed twice at room temperature (23°C) with 2.5X SSC, 0.1% SDS. Each of the membranes registered about 400 cpm

with the hand-held survey meter. Autoradiography (72 hours) of the 16 hybridized membranes was very informative. Each of the membranes contained many positive "light-ups". These 8 sets of lifts yielded a total of 35 strongly hybridized areas and 20-25 less strongly hybridized areas.

The next step of screening the libraries involved the isolation of the positively hybridized plaques from non-STR containing plaques. A total of 33 positively hybridized areas were selected and prepared for the secondary screening procedure. Each phage area (pick) was transferred, by the use of a borosilicate transfer pipet and a rubber bulb, into separate 1.5 ml microcentrifuge tubes which contained 500 µl of SM buffer and 20 µl of chloroform. After each pick had incubated at room temperature (23°C) for 2 hours, they were diluted 1:100 in SM buffer. Two different volumes, 10 and 100 µl, of the diluted phage picks were mixed with separate 200 µl volumes of XL1-Blue MRF' cells (see Materials and Methods) to create plates of phage with isolated plaques (ideally between 100 and 1,000 pfu/plate). Each of the 33 plates contained a small number of plaques; some as few as 50 and some as many as 1000. A Colony/Plaque Screen™ membrane was used on each plate and treated to denature and affix the DNA. All 33 membranes were hybridized at 53°C overnight with the 3 α-^{32}P end-labeled STR probes 3, 6 and 8. They were washed at room temperature twice with 2.5X SSC, 0.1% SDS. A Geiger counter survey indicated 300-500 cpm per membrane. All of the lifts were exposed to x-ray film for 48 hours.

One plate did not contain any positively hybridized plaques while ten plates had weakly hybridized positive "light-ups". All other plates contained several plaques which were moderately to very strongly hybridized. A set of 15 samples was chosen for a

tertiary screen of the plaques. Isolated plaques were placed into 500 µl of SM buffer with 20 µl of chloroform. The tubes were incubated at room temperature (23°C) for 3 hours. Each of the phage samples was diluted 1:100 in SM buffer from which a 5 µl volume was incubated with 200 µl XL1-Blue MRF' cells to create a new tertiary phage plate. Some of the plates contained 5 plaques, whereas others contained 200 plaques. A separate Colony/Plaque Screen™ membrane was used on each of these plates. After the DNA was denatured and affixed, the membranes were hybridized with α-^{32}P end-labeled STRs -3, -6 and –8. The membranes were washed at room temperature twice with 2.5X SSC, 0.1% SDS. All of the membranes were exposed to x-ray film for 48 hours.

Isolated plaques were selected and transferred to tubes with SM buffer and chloroform from 13 of the 15 plates created for the tertiary screening. Two of the plates did not have positively hybridized plaques distinctly isolated from other plaques, and were therefore not selected. Isolated plaques were selected from 14 of the 17 plates which had only been processed through the secondary screening procedure. The other 3 plates did not have isolated and positively hybridized plaques.

Twenty-seven isolated positively hybridized plaques were selected and transferred to tubes containing SM buffer and chloroform. Each of these clones was processed through the *in vivo* excision protocol (see Materials and Methods) in order to create the phagemid form of the ZAP Express® clones (which can be grown in XLOLR bacterial cells and processed through a rapid plasmid isolation procedure). Only 2 of the clones did not result in transformed XLOLR bacterial cells growing on an LB-kanamycin (50 µg/ml) plate. From most of the remaining 25 plates, isolated bacterial colonies were chosen for inoculation into LB-tetracycline (12.5 µg/ml), incubation at 37°C overnight,

followed by rapid plasmid isolation. Since some of the plates did not have isolated colonies, 3-5 colonies were chosen to be inoculated for a rapid plasmid isolation.

Twenty-five rapid plasmid isolations were performed from the XLOLR cells. A Stratagene® technical support person informed me of the possibility that cryptic DNA strands may be formed from phagemids grown in XLOLR cells. Since cryptic DNA molecules could result in anomalous DNA sequence patterns, each of the isolated plasmid samples was diluted to about 5 ng/µl and used to transform MRF' competent cells. The transformed MRF' cells were grown on agar plates of LB tetracycline (12.5 µg/ml)/kanamycin (50 µg/ml) in order to maintain the F' episome and the phagemid clone. An isolated colony was chosen from each of the transformed MRF' plates and used to inoculate separate 5 ml cultures of LB medium. Each of the cultures was processed through the rapid plasmid isolation procedure and the DNA samples were resuspended in 40 µl TE with RNase A. A 2 µl volume of each plasmid sample was loaded on a 1% agarose gel and electrophoresed at 100 V for 2.5 hours (Figs. 57 and 58).

Each of the plasmid samples was screened for the presence of an STR locus by beginning with a Southern (1975) transfer of the plasmid DNA samples from the agarose gels. The membranes containing the DNA samples were then hybridized overnight at 53°C with the γ-^{32}P end-labeled probes PSTR-3, -6, and -8. They were washed at room temperature twice with 2.5X SSC, 0.1% SDS. Autoradiography of the membranes indicated the presence of STR loci in most of the DNA samples (Figs. 59 and 60).

The T3 and T7 sequencing primers were used to determine the DNA sequence of the cloned segment in every plasmid isolated. Some of the isolates (Δ22, [3]5 and [2]4)

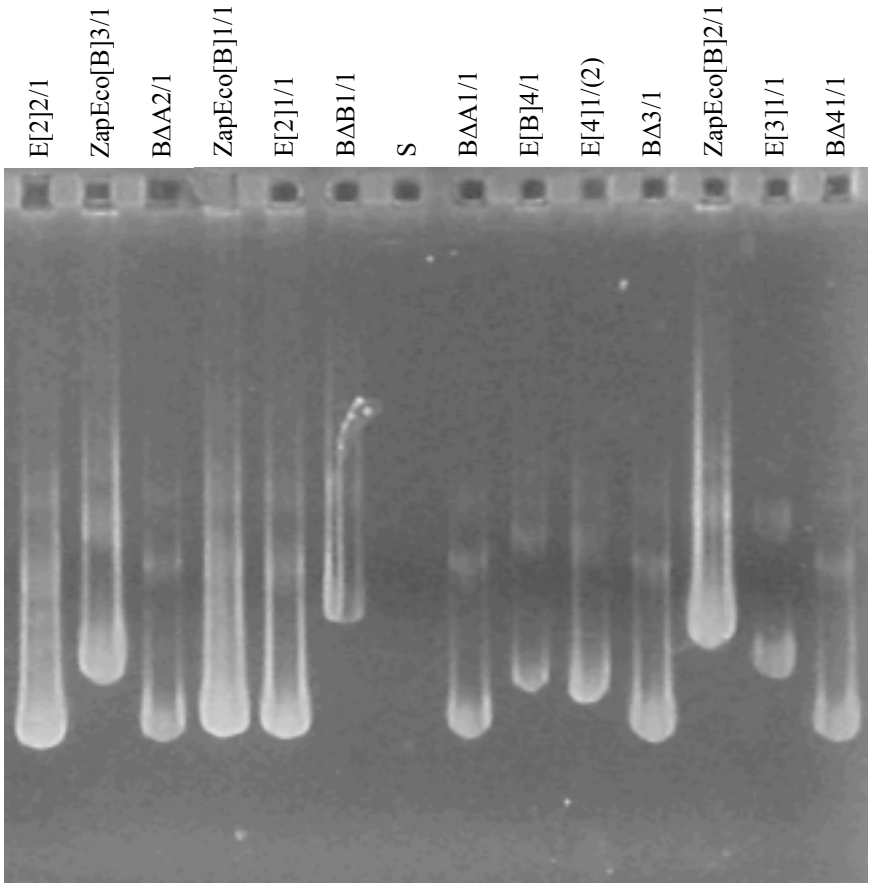

Figure 57. **Electrophoretic analysis of plasmids isolated from MRF' cells on 9/13/01.** The assigned clone number of each plasmid sample is indicated above each well, which is the same as on the autoradiograph (Fig. 59). The size standard "S" is the plasmid pBR322 digested with *Hin*fI, which migrated beyond the photographed region. This 1% agarose gel was electrophoresed at 100 V for 2.5 hours.

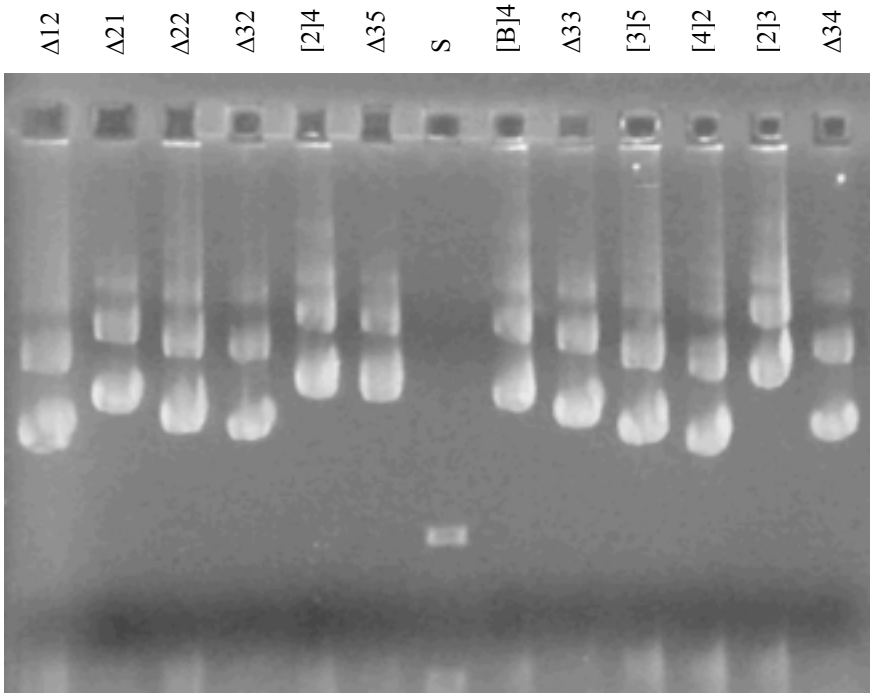

Figure 58. **Electrophoretic analysis of the plasmids isolated from MRF' cells on 9/28/01.** The assigned clone number of each plasmid sample is indicated above each well, which is the same as on the autoradiograph (Fig. 60). The size standard "S" is the plasmid pBR322 digested with *Hin*fI. This 1% agarose gel was electrophoresed at 100 V for 2 hours.

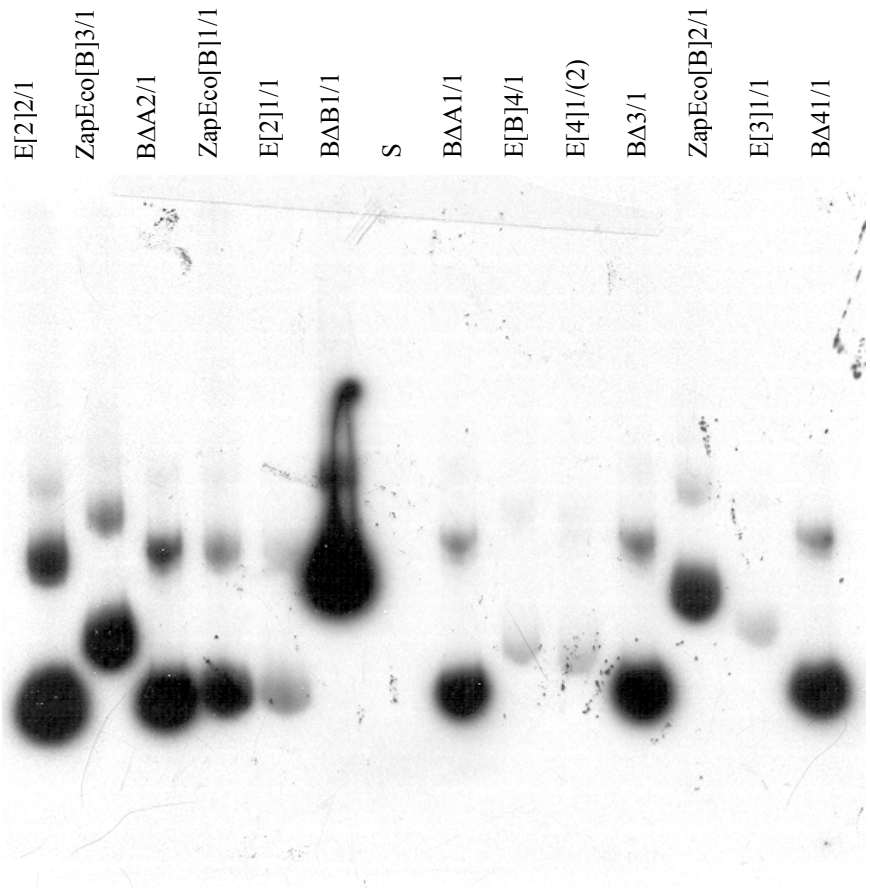

Figure 59. **Autoradiograph of plasmid samples from 9/13/01 which were hybridized with the γ-^{32}P end-labeled probes PSTR-3, -6, and -8.** The assigned clone number of each plasmid sample is indicated above each well, which is the same as in Fig. 57. The size standard "S" is the plasmid pBR322 digested with *Hin*fI, which migrated beyond the photographed region. Autoradiography proceeded for 48 hours.

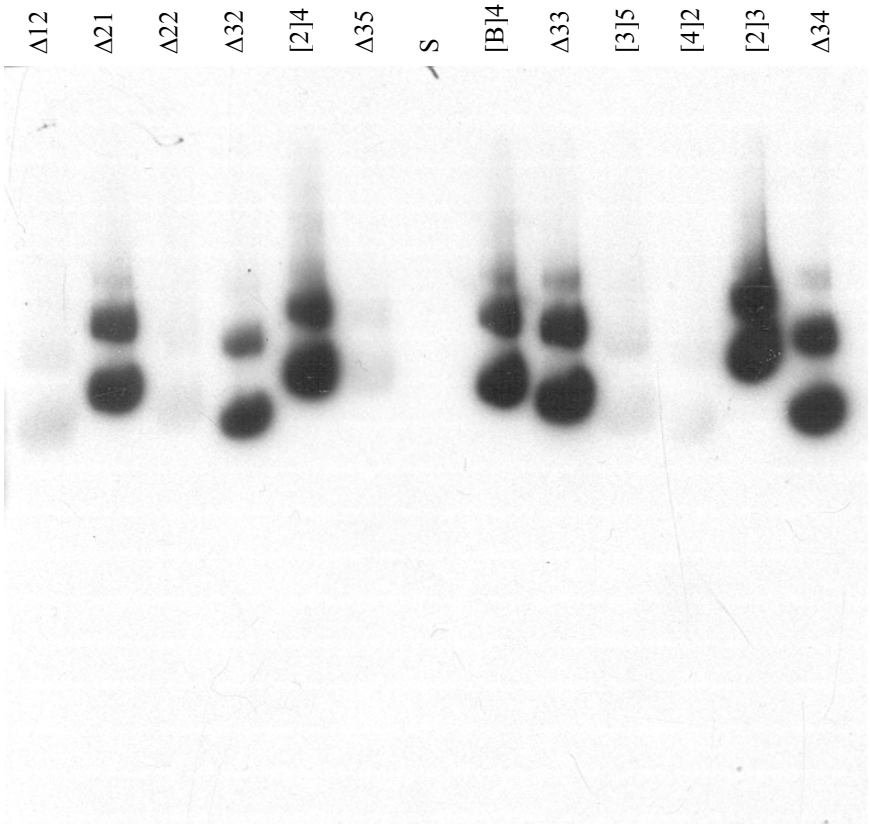

Figure 60. **Autoradiograph of plasmid samples from 9/28/01 which were hybridized with the γ-^{32}P end-labeled probes PSTR-3, -6, and -8.** The assigned clone number of each plasmid sample is indicated above each well, which is the same as in Fig. 58. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. Autoradiography proceeded for 3 hours.

obviously included 2 different plasmids because a mixture of 2 different sequence profiles was visible on the autoradiograph. Clone [3]5 appeared to have an STR region with the motif $(GAT)_n$, but it was obscured by a contaminating sequence profile. The original plasmid DNA from each of these 3 complex isolates was used to transform competent MRF' cells again. Isolated colonies (8 from each plate) were then selectively plated on an agar (master) plate and used as a substrate for a colony PCR reaction using the primers T3 and T7. The PCR products were eletrophoresed on a 2% agarose gel and the sizes were compared (data not shown). Two different sizes of DNA inserts were identified by the colony PCR procedure from the plate of clone [2]4. An estimation of size, compared to the plasmid DNA which was positively hybridized on the Southern (1975) (Fig. 60), was used to determine which colony carried the desired phagemid clone. A colony with a larger insert, about 1,900 bp instead of 1,100 bp, was then selected from the master plate and used for plasmid isolation procedures. The plasmid was then subjected to a rapid plasmid isolation procedure and processed through the DNA sequencing protocol.

Only one size of DNA insert was observed from PCR amplified colonies of the clone Δ22 and clone [3]5. The isolated plasmids from these clones evidently did not carry an STR since the probe did not bind to the plasmids following Southern (1975) transfer (Fig. 60). However, it was obvious that an STR was present in the clone [3]5 from the mixed DNA sequences on the autoradiograph. Additionally, the size of the PCR amplified DNA insert (400 bp) was relatively small. Since another clone was possibly present, but not yet obtained, another set of 8 colonies from the plate of transformed cells from clone [3]5 were PCR amplified. From this set, 2 of the clones appeared to have

large (about 2,000 bp) products, while the other 6 were still about 400 bp. The colony which carried the larger DNA insert was processed through a rapid plasmid isolation and the DNA sequence was obtained (see below). Another set of colonies (5) of clone Δ22 were processed by colony PCR. All of the amplified samples contained the same size insert as those seen with the first set of 8 colony PCR products from clone Δ22.

Primers were designed for PCR amplification of each STR locus as soon as the complete DNA sequences of the flanking regions became available. The phagemid clone ZapEco[B]1/1 encoded the STR locus: $(GAT)_{11}$. The following primers were designed to amplify this locus:

ZEB1/1F2: 5'-GCACTGTGCATCACTTATTCG-3'

ZEB1/1R2: 5'-AGCAGGCAGCTAAAAACCAC-3'

The expected product size was 206 bp. Amplifications of this locus with these primers were performed at 52, 55 and 58°C annealing temperatures. The amplified products of the 52 and 55°C amplifications were loaded on a 7.5% (37.5:1) polyacrylamide gel and electrophoresed at 200 V for 4.5 hours (Fig. 61). A distinct single band was observed at about 200 bp from each of the amplified samples and at all annealing temperatures. For example, the PCR amplifications at 58°C produced products which appeared very similar to the 55°C products (data not shown). These results of these experiments indicate that this locus is not likely to be polymorphic. However, the secondary banding pattern (faint bands) produced at 55 and 58°C appeared to have some distinguishing characteristics (polymorphic bands) which may prove to be useful in future DNA typing experiments.

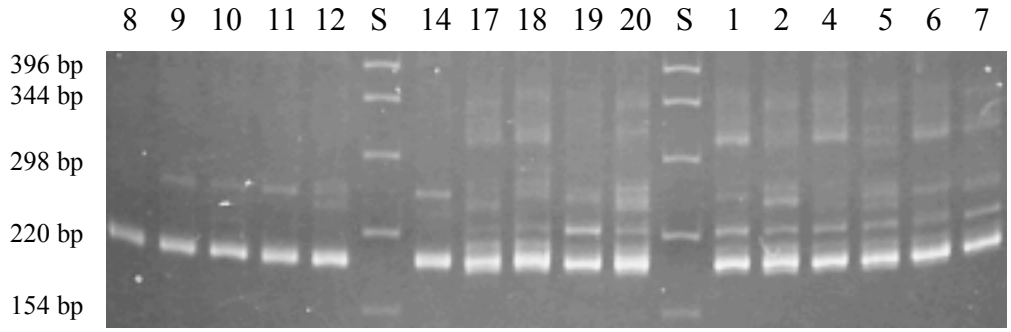

Figure 61. **Electrophoretic analysis of ZE[B]1/1F2/R2 amplified Spoonbill genomic DNA.** The Spoonbill samples are indicated above each well. The expected product size was 206 bp. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. This 7.5% (37.5:1) polyacrylamide gel was electrophoresed at 200V for 4.5 hours.

The clone E[2]1/1 encoded the STR locus: $(GGAGA)_9GGCGA(GGAGA)_{15}$. The presence of the point mutation adenosine to cytidine in the middle of the STR locus was discouraging. A single mutation in the center of an STR locus provides a focal point, or reference, which may prevent slippage during replication and therefore reduce change over time. The following 2 primers were designed to amplify this locus:

E21/1F2: 5'-AATTACTCAGGGAGAGGAG-3'

E21/1R: 5'-CATCTGCTGCTGACCCAAG-3'

Because of the location of a *Tsp*509I (AATT) restriction endonuclease site, the forward primer, E21/1F2, had to be designed with the restriction site included (underlined), as well as the first 9 nucleotides of the STR locus. The expected size of the PCR product was 175 bp. This locus was PCR amplified using 52, 58 and 60°C annealing temperatures. The 52°C temperature appeared to amplify the largest fragments (500 and 800 bp bands) preferentially. The products amplified at 60°C were loaded on a 7.5% (37.5:1) polyacrylamide gel and electrophoresed at 200 V for 5 hours (Fig. 62). The expected product of 175 bp was not visible. However, the multiple bands produced between 500 and 600 bp appeared to have some unique characteristics. Future study of these products may thus identify a polymorphic locus.

The clone E[2]2/1 encoded the STR locus $(GGA)_7$. The presence of a *Tsp*509I site near one side of the STR locus caused a modification of the primer design in order for them to bind at approximately the same annealing temperature. The following primers had a melting temperature of about 53°C:

E221F2: 5'-TTTTATTTTCTCTTCCTACCTG-3'

E221R2: 5'-AATGTCTAGGGAAACAGAGG-3'

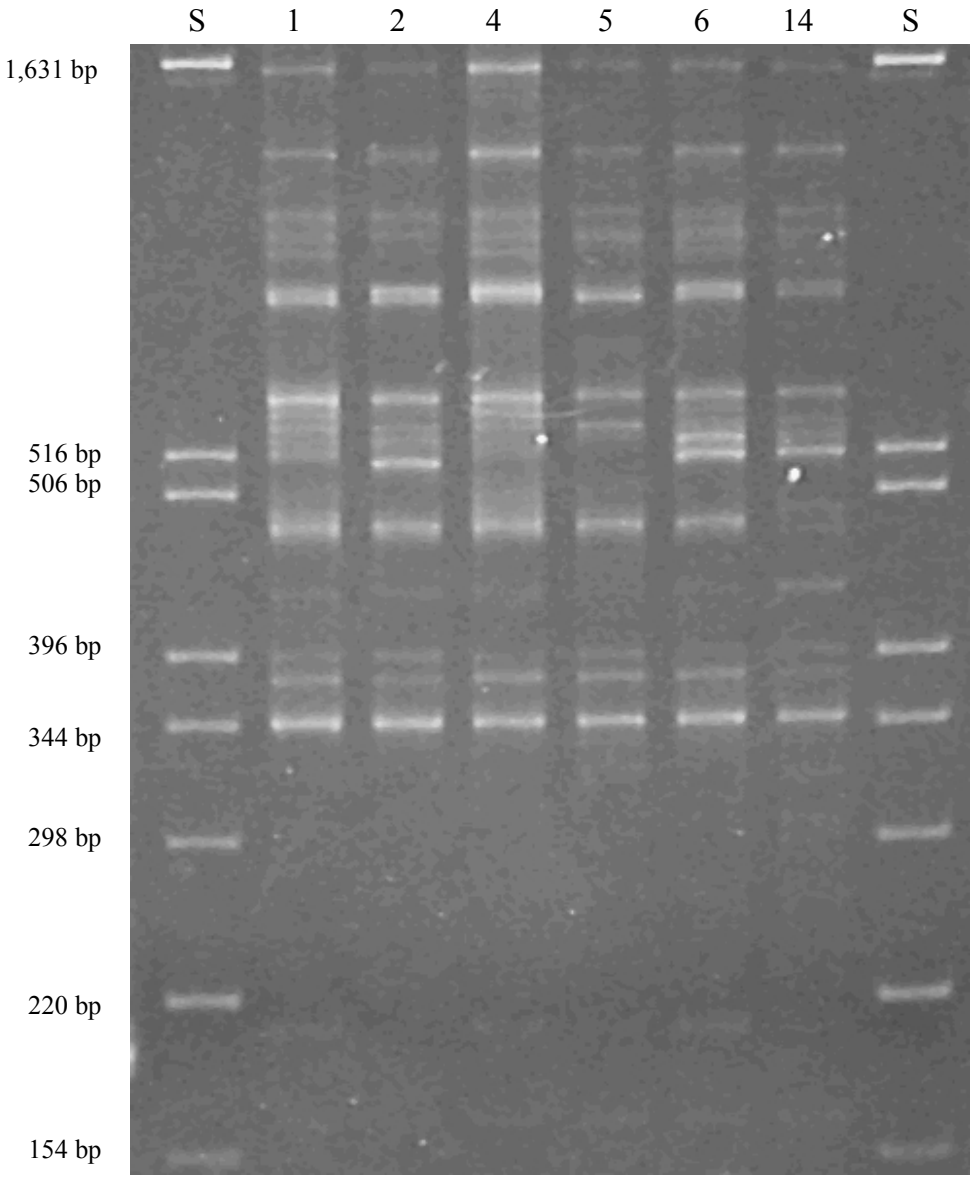

Figure 62. **Electrophoretic analysis of E21/1F2/R amplified Spoonbill genomic DNA.** The Spoonbill samples are indicated above each well. The expected product size was 175 bp. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. This 7.5% (37.5:1) polyacrylamide gel was electrophoresed at 100V for 10 hours.

The expected size of the amplified product was 199 bp. This primer set was used to amplify Spoonbill genomic DNA at 51 and 53°C annealing temperature. At both temperatures a specific band was produced at about 200 bp, as well as a less distinct band at about 280 bp (data not shown). Two less distinct bands were produced at about 230 and 250 bp when amplified at 51°C. The amplification of the same distinct products from different Spoonbill samples supported the fact that this locus must be considered non-polymorphic.

The plasmid DNA isolated from clone [3]5 was obviously a mixture of 2 different clones because of the noted presence of 2 DNA templates in the sequencing reactions. A transformed colony from the diluted plasmid DNA of clone [3]5 was identified, via colony PCR, as having a larger DNA insert than most of the other clones (see above; only 2 out of 16 colonies appeared to have the larger DNA insert). The plasmid DNA was isolated from the clone carrying the larger DNA insert. The DNA sequence of this clone encoded the STR motif $(GAT)_{16}(TAT)_4$ (Fig. 63). The following 2 primers were created:

3s5A: 5'-GGCTGAACACTGTTGTGCTCT-3'

3s5B: 5'-GAACCAAGCCTCCCTGAATA-3'

The expected size of the PCR product was 200 bp. Six Spoonbill genomic DNA samples were PCR amplified. The products were loaded on a 2% high resolution agarose gel (Sigma™) and electrophoresed at 80 V for 2 hours (data not shown). The banding pattern was not very distinct, but it appeared that 3 bands of different sizes were present among the 6 samples. This locus was considered to be polymorphic and the primer set was used to amplify additional Spoonbill genomic DNA samples.

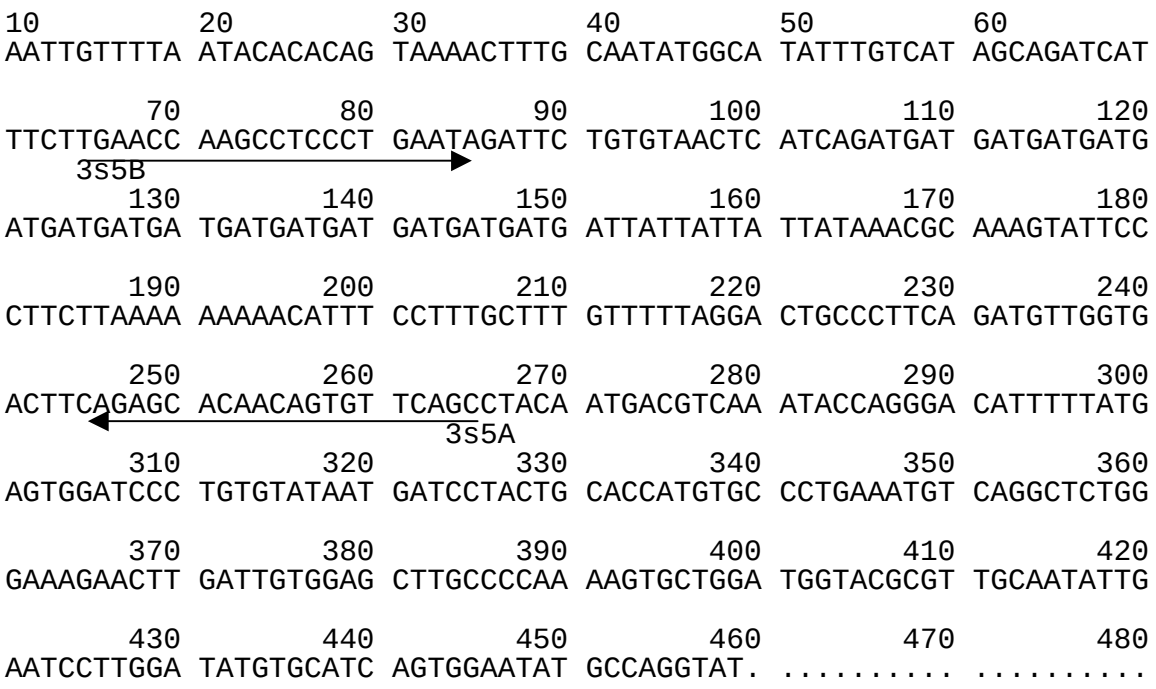

Figure 63. **Nucleotide sequence of STR locus Aajμ5.** The oligonucleotide primers 3s5A and 3s5B and their relative directions are indicated by the arrows. This clone has the GenBank accession #AF469478.

The clone [B]4 encoded the STR locus $(GAT)_8$. Two primers were designed to amplify this locus:

Bs4F: 5'-CGAGCAGCCAATAGACACTG-3'

Bs4R: 5'-CAGCAAACCTGACAAGAGCA-3'

The expected size of the amplified product was 203 bp. The amplifications were performed at 58°C and 61°C annealing temperatures. Only the 58°C temperature resulted in products (data not shown): multiple bands from 290 bp up to 1,300 bp, were present in 5 different samples. The banding pattern exhibited by this primer set was considered to be polymorphic, but not easily useful in differentiating Spoonbills.

The clone [2]3 encoded the STR $(AAT)_3(GAT)_7$. Two primers were designed to amplify this locus:

2s3F2: 5'-CCATACGACGTCATGCTCAG-3'

2s3R: 5'-CACGGGTCGAGATAAAGACAG-3'

The expected size of the amplified product was 212 bp. The PCR reactions were carried out at 58°C, 60°C, and 62°C annealing temperatures. A distinct band was visible at about 220 bp after the products were loaded and electrophoresed on a 7.5% (30:1) polyacrylamide gel (data not shown). Each of the product bands appeared to be the same size. Therefore, this locus was considered to be non-polymorphic.

Some of the other clones had some interesting characteristics, many of which proved difficult to analyze. One such clone was BΔB1/1. It encoded the motif (GGGGCACCAGTAAGGCACTCCCAGCAGCTCT), which appeared to repeat at least 13 times. It did contain a point mutation within some repeats, but it was otherwise very conserved. The DNA sequence of the complementary strand was determined (by primer

walking sequencing) up to 1,200 bp before the sequencing profile became too disrupted to read, possibly because of a contaminating clone. An amplification of plasmid isolate BΔB1/1 DNA with primers T3 and T7 resulted in two bands; 800 and 1,500 bp, which was unexpected. An amplification of the same DNA with the primers T3 and BΔBF3 (an internal primer designed to bind at 1,200 bp from the complementary end) resulted in a product of 1,900 bp. This product size was much larger than expected. It was unclear as to how the clone BΔB1/1 was created. Among the DNA sequences obtained from BΔB1/1, no STR region was identified as complementary to the STR probes. Additional experiments will be performed in attempts to determine the DNA sequence of the entire inserted DNA or at least identify the flanking regions of the large STR.

Many of the clones contained long regions (400 to 600) of CT-rich sequence. Most of these clones began their sequence at the MCS with the motif $(CTT)_5$ and were identical for the first 400 to 500 bp. The clones which contained this element were BΔA1/1, BΔA2/1, BΔA3/1, BΔ41/1, Δ21, Δ32, Δ33 and Δ34. No primers were created to amplify any part of these clones.

Clone E[3]1/1 was interesting because the T7 primer was used to determine the nucleotide sequence up to 150 bp, where it abruptly ended at an apparent compression artifact. Amplification of this clone with the primers T3 and T7 yielded a product of about 2,100 bp. Restriction endonuclease digestion with *Eco*RI of the amplified product, followed by 2% agarose gel analysis, showed the presence of 2 bands of about 1,000 and 1,200 bp. Determination of the DNA sequence from the complementary strand (by use of the T3 primer) would have to proceed through almost 1,800 bp in order to identify the STR. Therefore, the cloned DNA was submitted to Lone Star Labs, Inc. (lslabs.com) in

an attempt for them to determine the nucleotide sequence past the potential STR and through the flanking nucleotide sequence. Their method of determining the DNA sequence of a plasmid (using Big Dye™ terminators and an ABI PRISM 377™, Perkin-Elmer, automated DNA sequencer) through this region was not successful. The DNA sequence determinations, by Lone Star Labs, Inc., of 2 other clones, 3Δ5 and ZapEco[B]3/1, were also inhibited by a nucleotide compression (ZapEco[B]3/1) or the STR motif $(GGAGA)_n$ (3Δ5). Additional attempts by the Lone Star Labs personnel to sequence through these regions by optimization of the sequencing reactions were not entirely successful, although they did manage to add 40-50 bases to the total DNA sequence.

The 3' sequence of clone E[3]1/1, using the T7 primer, is $(GGAGA)_9GGA$. The total number of repeats at this locus is unknown, but is expected to be more than 10 repeats. Since the plasmid was not positively hybridized (Fig. 59), only the first 600 bp of the complementary DNA sequence was determined by the use of the primer T3. No visible STR locus, other than the GGAGA, was present. Primers were created within the flanking regions of the clone in an attempt to amplify the STR locus. A specific product was not amplified from genomic DNA. Therefore, the restriction endonuclease site within this clone is most likely a result of 2 DNA fragments ligated together and not the actual genomic nucleotide pattern. Additional DNA sequence determinations will be performed in order to read past the restriction endunulease site (*Eco*RI), and primers will be designed to attempt amplifications of the STR locus.

The 3Δ5 clone encoded a large and unusual double STR motif. The sequence from this clone was obtained with the T7 primer and was unique because it had about 17

repeats of CCTCT with one point mutation, followed immediately by over 15 repeats of GGAGA. The two sequences produce a large palindrome. An amplification with the T3 and T7 primers indicated that the overall size of the cloned fragment was about 2,000 bp. An effort to determine the DNA sequence from the fragment's complementary strand was begun using the T3 primer However, only about 100 bp of sequence could be obtained before the profile was obscured by sequencing artifacts. Nevertheless, a primer from each flanking sequence was designed:

3d5F: 5'-ACTCAGCCAAGGACTGATGG-3'

3d5R: 5'-GGAGCTGCCAAACAGTTTTTA-3'

Because of the difficulties encountered with obtaining adjacent downstream flanking sequences, the reverse primer 3d5R was designed to bind over 1500 bp from the STR. Although a *Tsp*509I site is adjacent to this site, the clone is of a *Sau*3AI digestion and so site should be a normal part of the Spoonbill genomic DNA. Since the primers were designed to bind within 100 bp of the clone ends, the expected size of the amplified DNA was 1,800 to 2,000 bp. A polyacrylamide gel analysis (7.5%, 30:1) indicated the presence of distinct bands at about 220, 400 and 1500 bp, with some less distinct products amplifying as well (data not shown). The anticipated amplicon was not observed on the gel. Although the size of theis STR made it a likely candidate to be a polymorphic locus, it is obvious that its GC-rich palindromic nature make DNA sequence and PCR analysis of the site very difficult. Further DNA sequence analysis of this locus will be required in order to determine if it is polymorphic.

The clone E[4]1/2 encoded the STR locus $(GGATA)_n$. Only the distant nucleotide sequences from the flanking regions were obtained. Two primers were designed to amplify this STR:

E4s12F: 5'-GCAGTCTTGGCCTTATGTGG-3'

E4s12R2: 5'-TAGGACAACGCAACAAGG-3'

Since the entire STR region was not visible (only 20+ repeats) on the autoradiograph, the exact size of the amplified product was not known. Therefore, these primers were used to amplify 2 ng of the plasmid DNA at 60°C. A product of about 450 bp was created. When the same primers and temperature were used with genomic DNA samples, products were less than 100 bp (most likely primer dimers). Either the amplification conditions needed to be adjusted, or one of the primers may need to be re-designed. The status of this locus as polymorphic is inconclusive, but most likely is a cloning artifact.

The ZapEco[B]3/1 clone encoded the locus $(GAT)_7$. Areas of compressed sequence flanked both sides of this motif, with one of the compressed sequences beginning with the motif $(AGG)_4$ before the sequence pattern became unreadable. A primer set could not be designed to flank the compressed sequence next to the STR $(GAT)_7$. Therefore, 2 primers were designed to amplify through the $(AGG)_4$ compressed sequence:

ZEB31F4: 5'-ATACACGCTGGAGCCAACAG-3'

ZEB31F4R: 5'-ATCCCCGAAACGAGCAATG-3'

The expected size of the amplified product was 140 bp. The amplification was carried out using a 60°C annealing temperature. The products were loaded and electrophoresed

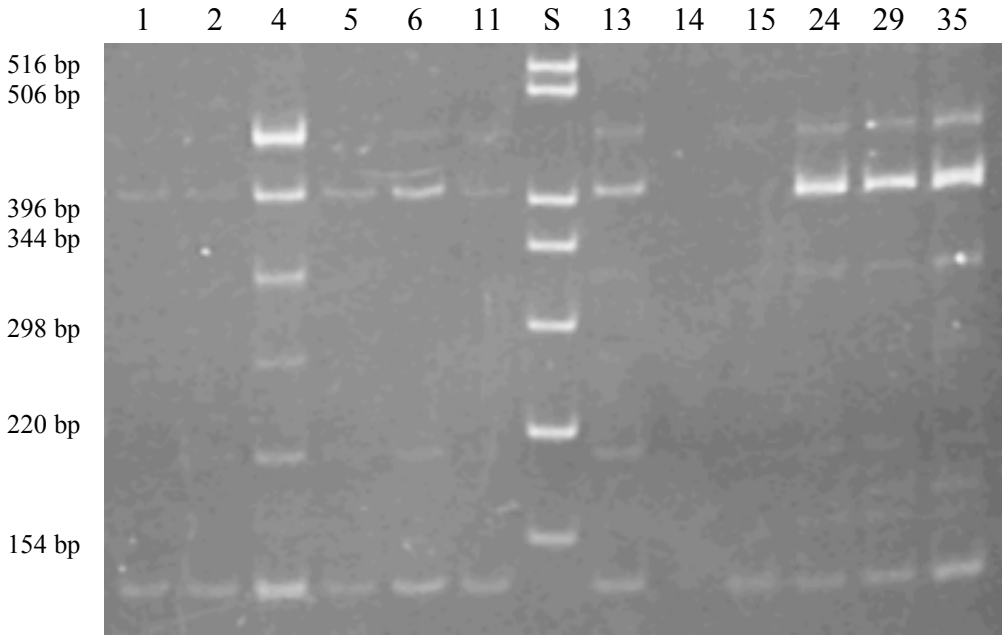

Figure 64. **Electrophoretic analysis of ZEB3/1F4/F4R amplified Spoonbill genomic DNA.** The Spoonbill samples are indicated above each well. The expected product size was 140 bp. The size standard "S" is the plasmid pBR322 digested with *Hin*fI. This 7.5% (37.5:1) polyacrylamide gel was electrophoresed at 100V for 10 hours in 0.5X TBE.

(100 V, 10 hours) on a 7.5% (37.5:1) polyacrylamide gel (Fig. 64) and distinct bands were visible at about 140 bp and 400 bp. However, the same distinct bands were obtained from each of the amplified Spoonbill samples, and thus this locus was considered to be non-polymorphic.

The E[B]4/1 clone encoded an STR, (GGAGA), which was repeated over 30 times. However, the locus could not be amplified since one end of the STR region was adjacent to the MCS. The numerous loci containing the STR motif $(GGAGA)_n$ continue to be of interest since they generally appeared to be very good polymorphic candidates. Similar polymorphic loci were found in other bird species. The STR locus Ccμ02 encoded the repeat motif $(GAGAA)_{23}$ and was discovered in the common cuckoo (*Cuculus canorus*) exhibiting 18 different alleles amongst 159 adults (Gibbs *et al.*, 1998). The STR locus *Dpμ*05 contains the repeat motif $(GAAGA)_{21}$ and was discovered in the yellow warbler (*Dendroica petechia*) exhibiting 46 alleles amongst 41 warblers (Dawson *et al*., 1997). At this time, none of the loci containing the STR motif $(GGAGA)_n$ has been characterized as polymorphic.

Characterization of polymorphic STR loci

PCR primers described previously were used to amplify the 5 polymorphic loci from more than 50 individual Spoonbill genomic DNA samples. The accurate identification of product sizes was critical to (1) quantify the total number of alleles existing for each locus (allelic diversity) and, (2) specify the allelic complement of each Spoonbill, so that individuals could be differentiated more powerfully. A variety of methods have been used to characterize polymorphic loci in many different species (Kijas *et al*., 1994; Luikart *et al*., 1997; Prodöhl *et al*., 1996). The number of samples to be screened, and the resources available to our laboratory, were considered when deciding upon the best method of characterizing multiple alleles at multiple loci. The accurate identification of amplified alleles was performed by gel electrophoresis and the results reviewed to determine if the method of allelic identification was viable.

Since the advent of capillary electrophoresis, sizes of amplified products could be determined quickly and with very little labor expenditure. Therefore, this method has become the standard method of allelic determination of most human STR loci. In an attempt to quickly analyze the Spoonbill alleles, an STR-specific PCR primer was fluorescently-labeled on its 5'-end. Then, amplification of Spoonbill genomic DNA was performed with a primer set containing the fluorescent-labeled primer and a non-fluorescent primer. The amplified product was loaded in an automated capillary DNA sequencer (ABI PRISM™ 310 Genetic Analyzer), along with a human size standard. The resultant electropherogram allowed an estimation of allelic sizes (data not shown). However, identification of specific alleles was not as easily carried out as with human allelic analyses since the Spoonbill alleles were not part of the analysis software.

Therefore, amplified Spoonbill alleles were not differentiated by capillary-based automated sequencer detection.

The need to identify specific alleles from primer-labeled PCR amplified products was met by the use of denaturing polyacrylamide gel electrophoresis. Two types of denaturing gels, 6% (30:1) and 4% (19:1), were routinely used. The 6% gels were routinely used to differentiate single nucleotide differences in ^{35}S-labeled sequencing products. They were also used to determine allele sizes by comparisons of them to electrophoresed sequencing products. Radio-labeled (1 primer 5'-end-labeled with γ-^{32}P) PCR products from each locus were electrophoresed on the 4% and 6% polyacrylamide gels and were visualized by autoradiography. The 4% polyacrylamide gels were routinely used in Dr. Arthur Eisenberg's Human DNA Identity Laboratory (University of North Texas Health Science Center, Fort Worth, TX) to differentiate the alleles of human STR loci. The ability to clearly differentiate alleles had already been optimized in his laboratory on the 4% gels. Fluorescent-labeled primers were used to amplify products which were electrophoresed on the 4% gels and visualized by the use of a Hitachi FMBIO® II Fluorescent Scanner (Figs. 65 and 66).

Typically, each locus was analyzed separately by performing PCR reactions on a population of Spoonbills with one primer set, then electrophoresing the products on a gel and recording the results. However, 2 loci, Aajμ1 and Aajμ2, were PCR amplified together, a process known as multiplexing, since the expected PCR product sizes were 163 bp and 217 bp, respectively. This allowed both loci to be processed simultaneously (Figs. 65 and 66). The total number of alleles for each locus, and their specific sizes, were then determined for the populations under study.

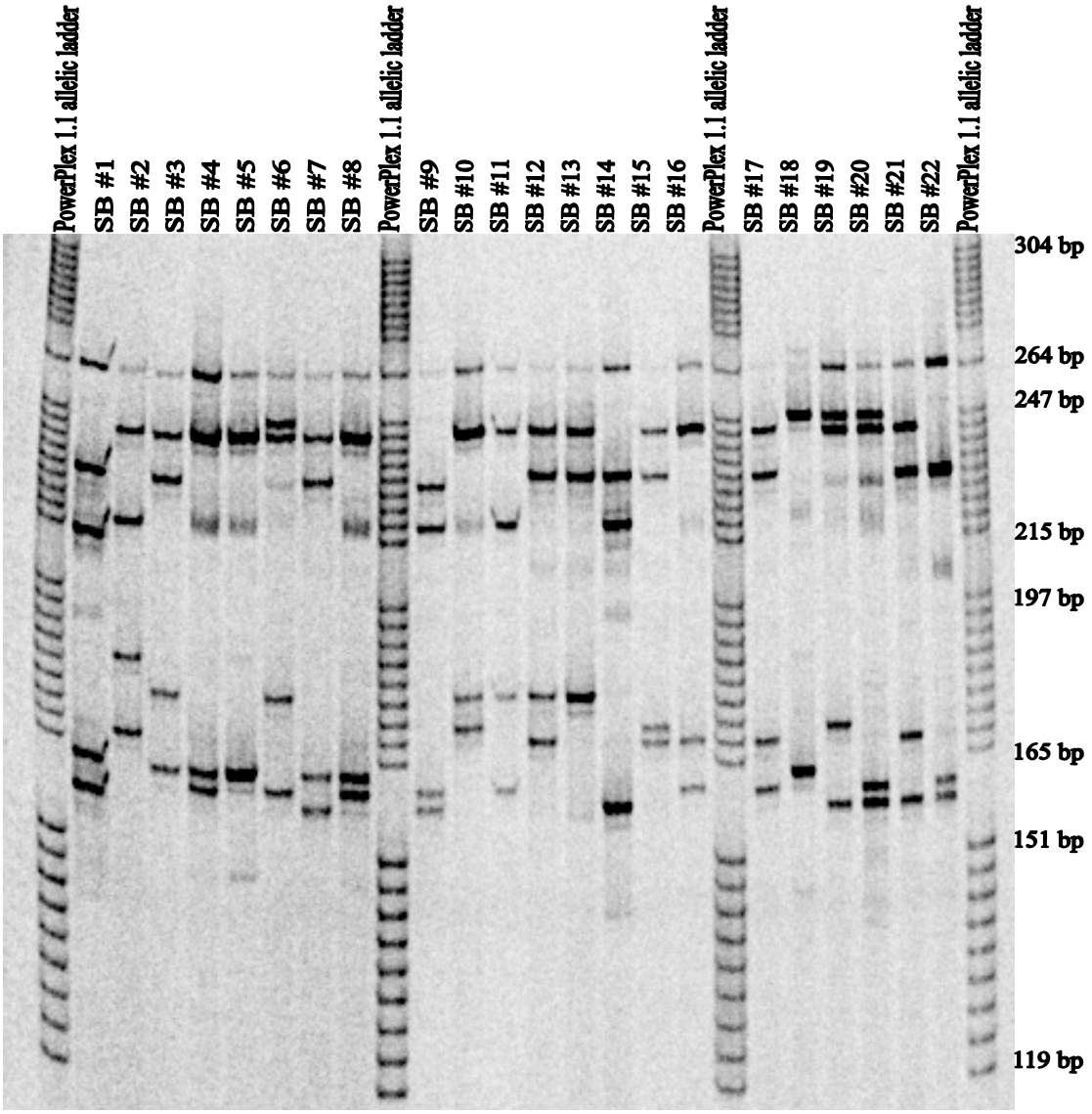

Figure 65. **Fluorescent image of the amplified products from the Aajμ1 (lower products) and Aajμ2 (upper products) polymorphic loci using 22 (Table IV) Spoonbill genomic DNA samples**.

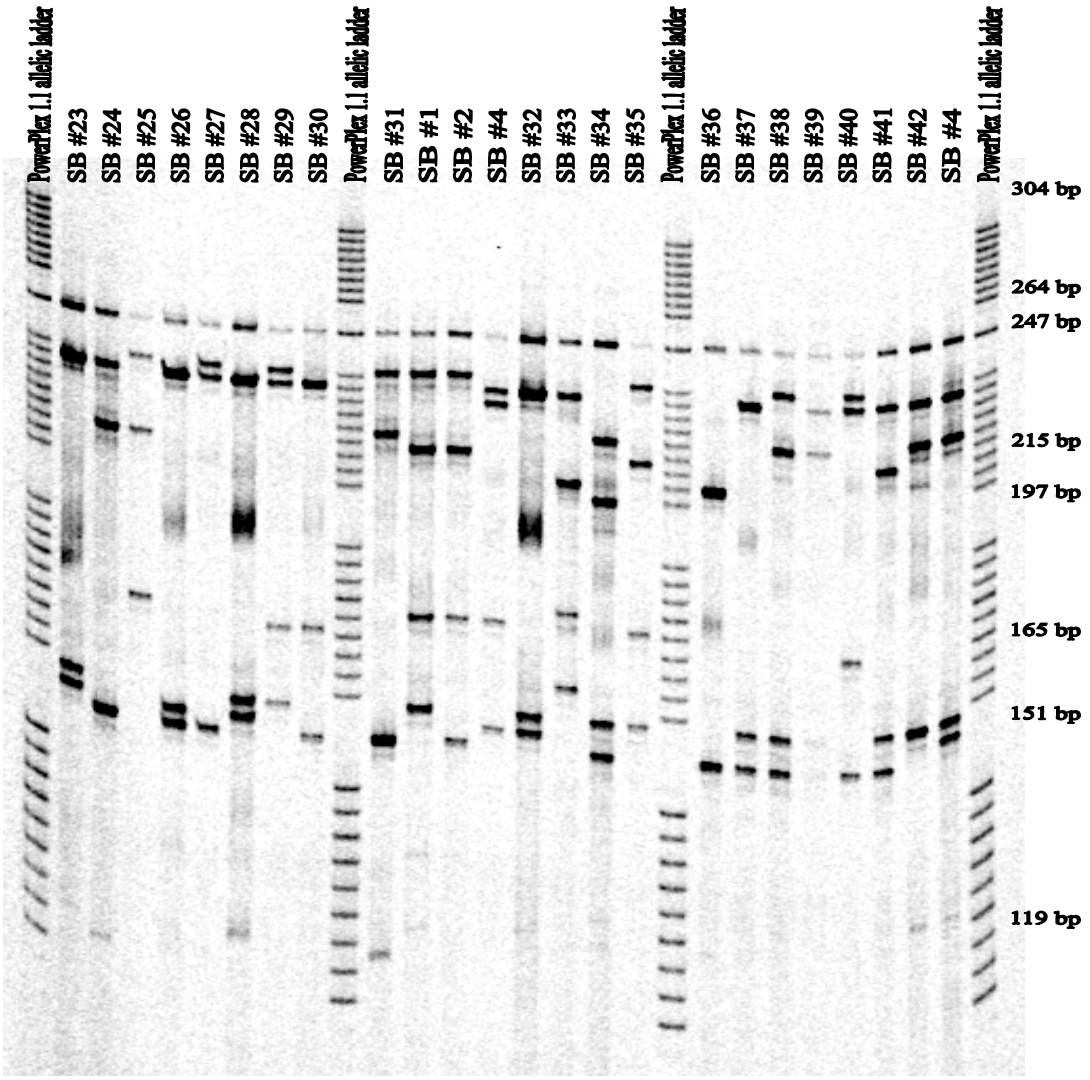

Figure 66. **Fluorescent image of the amplified products from the Aajμ1 (lower products) and Aajμ2 (upper products) polymorphic loci using 24 (Tables IV and V) Spoonbill genomic DNA samples**. Samples #1, #2 and #4 were loaded on this gel to allow comparisons of these products (#23-#42) with samples #1-#22 (Fig. 65).

The identification of allele sizes on the fluorescent images was facilitated by the use of the human STR ladder PowerPlex™ 1.1 Allelic Ladder Mix (Promega). This ladder was made to be specific for the alleles which were amplified in the human population. The sizes of the human alleles were known and could be correlated with the alleles created from the amplification of Spoonbill genomic DNA. Each allele of a particular locus was identified by the number of times the STR motif was repeated. The number of alleles, relative to 42 amplified Spoonbill samples, was noted.

Three additional loci, designated Aajμ3, Aajμ4 and Aajμ5, were discovered during attempts to isolate STR loci. These loci were identified as polymorphic by amplifying each from a variety of Spoonbill DNA samples and electrophoresing the products on non-denaturing polyacrlamide gels, routinely 7.5% (37.5:1). These gels allowed for greater resolution of the amplified products relative to agarose gels. The amplified products were electrophoresed and stained with ethidium bromide for detection purposes, which allowed for much quicker analysis of the products than by the use of radioisotopes and fluorescent-labeled primers. However, the resolution of DNA products in the 1.5 mm thick 7.5% polyacrylamide gels was not as good as the denaturing 0.25 mm thick 4% or 6% gels. Attempts to utilize the 4 or 6% gels in the same gel assemblies (20 cm x 20 cm x 1.5 mm) were not successful (data not shown). The thicker spacers (1.5 mm versus 0.25 mm) allowed for the creation of thicker gels, which could be more readily removed from the glass plates without tearing. However, the use of thicker polyacryamide gels resulted in decreased resolution of the amplified DNA products.

Since the thickness of the gels appeared to be more important than the acrylamide percentage, 4% denaturing gels were used to resolve amplified fluorescent-labeled

products of Aajμ1 and Aajμ2. However, hundreds of amplifications were performed on small numbers (1-16) of Spoonbill samples in attempts to optimize the PCR parameters. It was important to be able to analyze these products in our laboratory, without a fluorescent scanner, especially for any future work on Spoonbills or Flamingos. Therefore, the 4% polyacrylamide gel electrophoresis procedure was modified to be used in our laboratory. This was accomplished by loading the gels with the products of γ-^{32}P end-labeled primers and electrophoresing as directed (see Materials and Methods). Since the gel would be too thin to carefully remove from the glass plates and stain with ethidium bromide, one of the plates was treated with a gel binding solution (bind silane) to hold the gel firmly and precisely in place. To optimize the resolution of the DNA products, the bound gel was submerged in 10% acetic acid/12% methanol to leach out the urea. After the gel was allowed to dry on the plate for 1-2 hours at 60°C, it was wrapped in transparency acetate. An autoradiograph was produced from each gel in about 3-4 hours..

One additional set of Spoonbill DNA samples was received from the Sedgwick County Zoo in December 2001 (Table VI). Each sample was amplified with the primer sets specific for the Aajμ1 and Aajμ2 loci and electrophoresed on 4% gels as described above (Figs. 67, 68 and 69). One of the samples, #52, appeared to have the requisite number of alleles for the locus Aajμ1 (Fig. 67 and 68). However, upon analysis of the 4 alleles produced from the locus Aajμ2 (Fig. 69) it was apparent that sample #52 must have been contaminated, most likely with sample #51 since it contains 2 of the 4 alleles present in sample #52. Spoonbill #52 was discarded from any further analyses because its true complement of alleles was compromised.

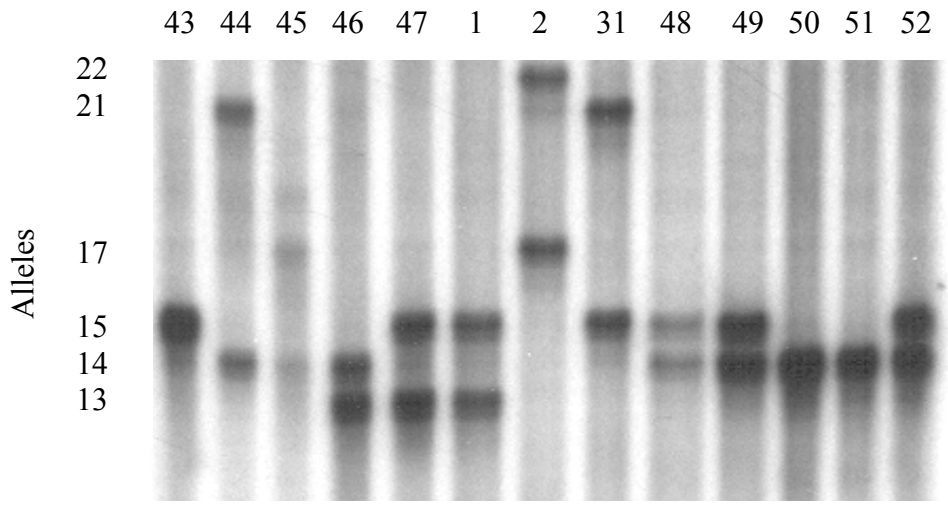

Figure 67. **Electrophoretic analysis of locus Aajμ1 amplification products 1, 2, 31, and 43-52.** The Spoonbill sample numbers are listed at the top of the figure and the allele designations are indicated on the left. Sample #52 shares 1 allele with sample #51, most likely because of sample contamination (see Fig. 69). Samples #1, #2 and #31 were loaded as the size standards since their sizes were identified in Fig. 66. This denaturing 4% polyacrylamide gel was electrophoresed at 50 W for 1 hour.

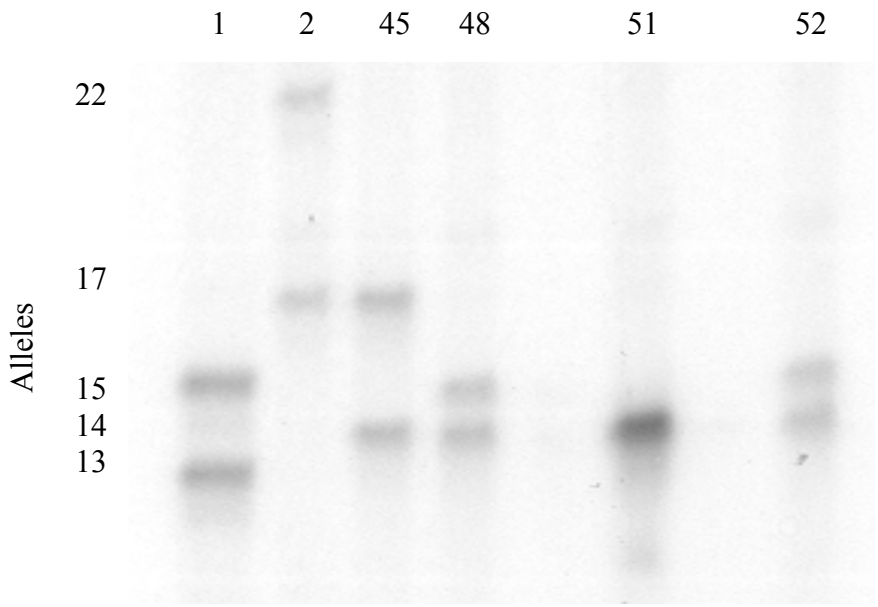

Figure 68. **Electrophoretic analysis of locus Aajμ1 amplification products 1, 2, 45, 48, 51 and 52.** The Spoonbill sample numbers are listed at the top of the figure and the allele designations are indicated on the left. This denaturing 4% polyacrylamide gel was electrophoresed at 50 W for 1 hour. Samples #1 and #2 were loaded as size standards (Figs. 66 and 67).

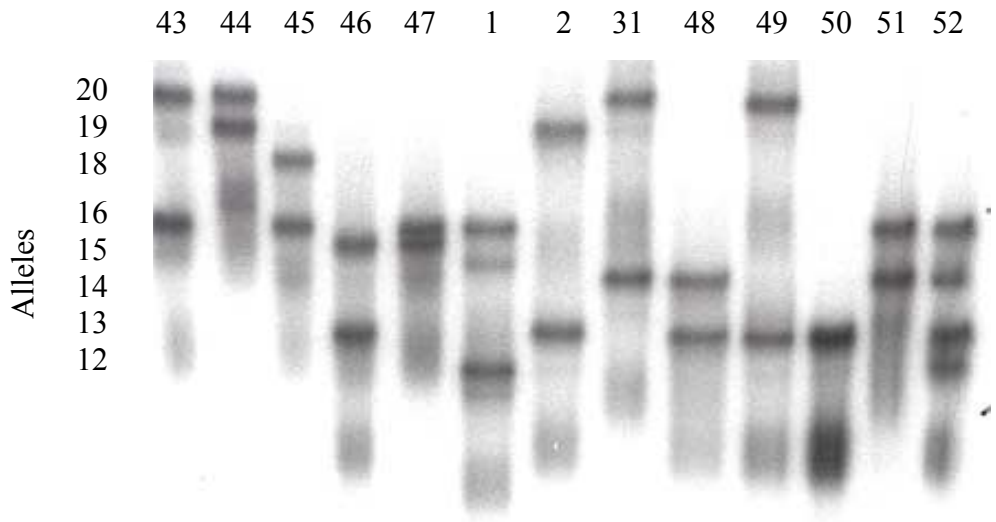

Figure 69. **Electrophoretic analysis of locus Aajμ2 amplification products 1, 2, 31, and 43-52.** The Spoonbill sample numbers are listed at the top of the figure and the allele designations are indicated on the left. Sample #52 shares 2 of 4 alleles with sample #51, most likely because of sample contamination. Samples #1, #2 and #31 were loaded as the size standards since their sizes were identified in Fig. 66. This denaturing 4% polyacrylamide gel was electrophoresed at 50 W for 1 hour.

PCR amplifications at 3 other loci (Aajμ3, Aajμ4, and Aajμ5) were performed with the 51 Spoonbills. Each set of products was electrophoresed on 4% polyacrylamide gels and autoradiographs were produced (Figs. 70, 71, 72, 73, 74, 75, 76 and 77). Some amplified products from each locus were electrophoresed on 6% DNA sequencing gels next to a DNA template which had been sequenced with ^{35}S (data not shown). From these autoradiographs the nucleotide sizes of each allele was determined and each allele was assigned a specific descriptor, which was relative to the number of times the STR motif was repeated. The alleles from each Spoonbill were compiled into Table XII and constituted the collection of DNA profiles for each of the 51 Spoonbills in this study.

For each locus, a table was created which contained the total number of alleles, the frequency of each allele, and the observed and expected heterozygosities (Tables XIII, XIV, XV, XVI, and XVII). Observed heterozygosity (H_O) was calculated by dividing the number of Spoonbills exhibiting allele heterozygosity by the total number of Spoonbills (51). The observed heterozygosities were as follows: Aajμ1 (0.75), Aajμ2 (0.75), Aajμ3 (0.59), Aajμ4 (0.51), and Aajμ5 (0.59). Expected heterozygosity (H_E) was derived from the allele frequencies of each locus and was calculated as $1-\sum(P_i^2)$, where P_i is the frequency of the *i*th allele (Luikart *et al*., 1997). The expected heterozygosities were as follows: Aajμ1 (0.80), Aajμ2 (0.76), Aajμ3 (0.57), Aajμ4 (0.38), and Aajμ5 (0.64). Only locus Aajμ4 showed a considerable difference (0.13, or 25%) between the observed (0.51) and expected (0.38) heterozygosities. The deviation between the observed and expected heterozygosities was because the locus is sex-linked. Since alleles 2 and 15 were present on the sex chromosomes, the degree of heterozygosity was dictated by the sex of each Spoonbill, not by the random assortment of alleles.

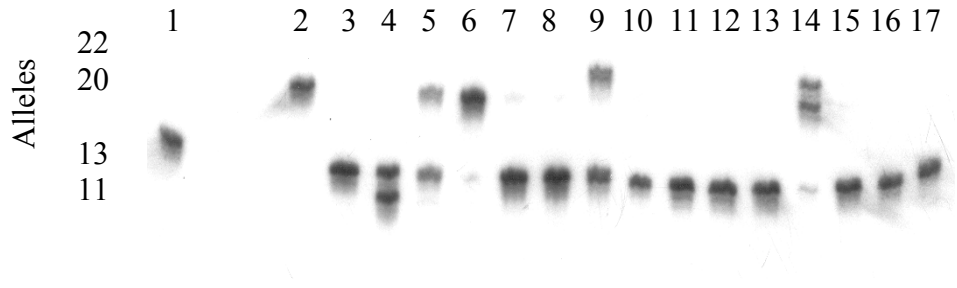

Figure 70. **Electrophoretic analysis of locus Aajμ3 amplification products 1-17.** The Spoonbill sample numbers are listed at the top of the figure and the allele designations are indicated on the left. This denaturing 4% polyacrylamide gel was electrophoresed at 50 W for 1 hour.

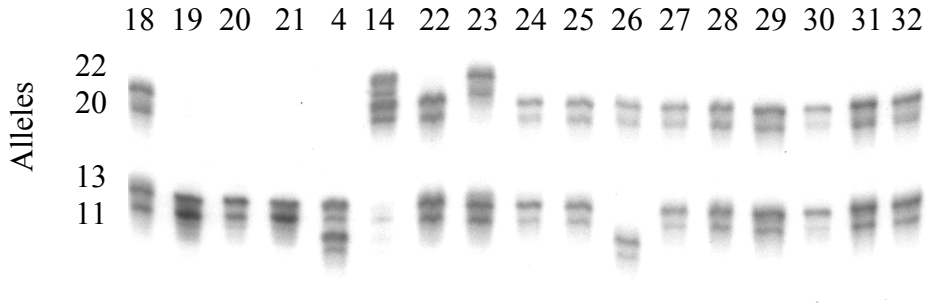

Figure 71. **Electrophoretic analysis of locus Aajμ3 amplification products 18-32.** The Spoonbill sample numbers are listed at the top of the figure and the allele designations are indicated on the left. Spoonbill samples #4 and #14 were used as size standards relative to Fig. 70. Each allele resulted in a stuttered image on the gel because of a problem during electrophoresis. However, determination of alleles was still possible. This denaturing 4% polyacrylamide gel was electrophoresed at 50 W for 1 hour.

Figure 72. **Electrophoretic analysis of locus Aajμ3 amplification products 33-42.** The Spoonbill sample numbers are listed at the top of the figure and the allele designations are indicated on the left. Spoonbill samples #4 and #14, loaded together and labeled 4/14, were used as size standards relative to Figs. 70 and 71. Each allele resulted in a stuttered image on the gel because of a problem during electrophoresis. However, determination of alleles was still possible. This denaturing 4% polyacrylamide gel was electrophoresed at 50 W for 1 hour.

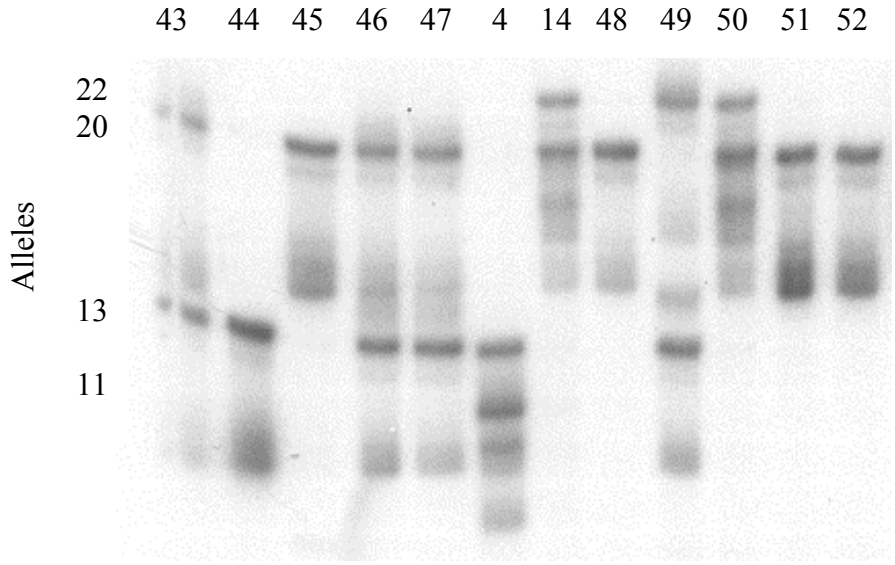

Figure 73. **Electrophoretic analysis of locus Aajμ3 amplification products 43-52.** The Spoonbill sample numbers are listed at the top of the figure and the allele designations are indicated on the left. Spoonbill samples #4 and #14 were used as size standards relative to Figs. 70, 71 and 72. This denaturing 4% polyacrylamide gel was electrophoresed at 50 W for 1 hour.

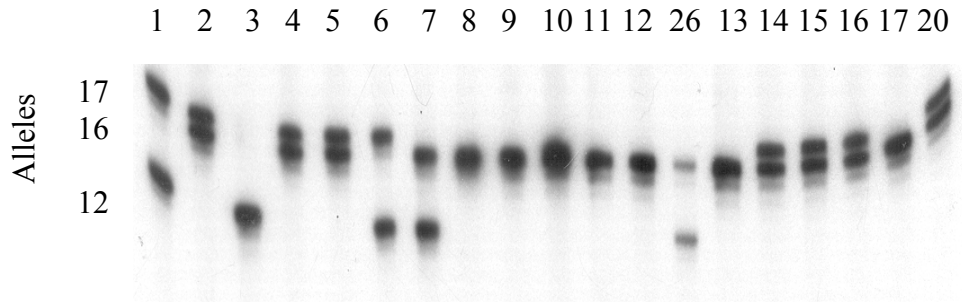

Figure 74. **Electrophoretic analysis of locus Aajμ5 amplification products 1-17, 20 and 26.** The Spoonbill sample numbers are listed at the top of the figure and the allele designations are indicated on the left. This denaturing 4% polyacrylamide gel was electrophoresed at 50 W for 1 hour.

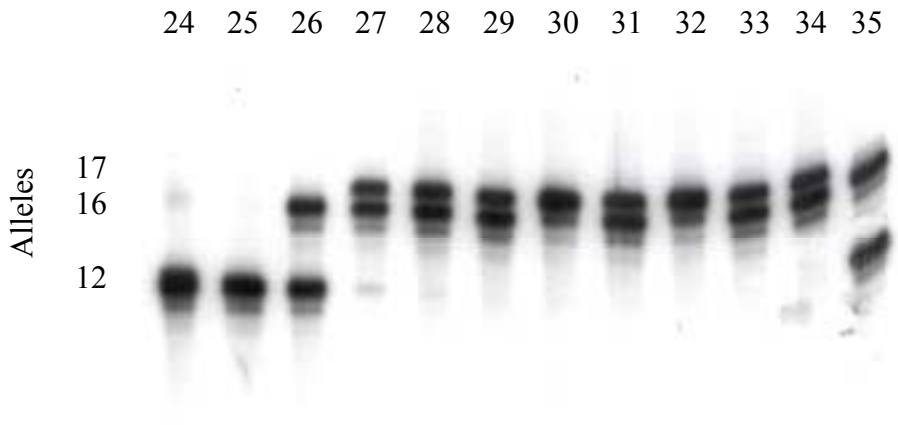

Figure 75. **Electrophoretic analysis of locus Aajμ5 amplification products 24-35.** The Spoonbill sample numbers are listed at the top of the figure and the allele designations are indicated on the left. This denaturing 4% polyacrylamide gel was electrophoresed at 50 W for 1 hour.

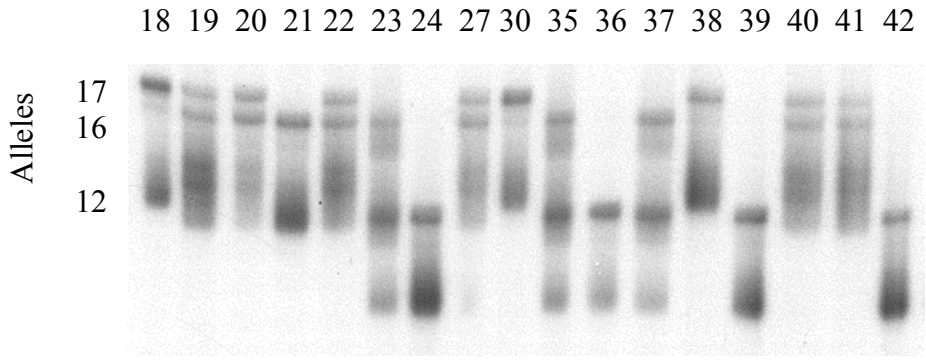

Figure 76. **Electrophoretic analysis of locus Aajμ5 amplification products 18-24, 27, 30 and 35-42.** The Spoonbill sample numbers are listed at the top of the figure and the allele designations are indicated on the left. This denaturing 4% polyacrylamide gel was electrophoresed at 50 W for 1 hour.

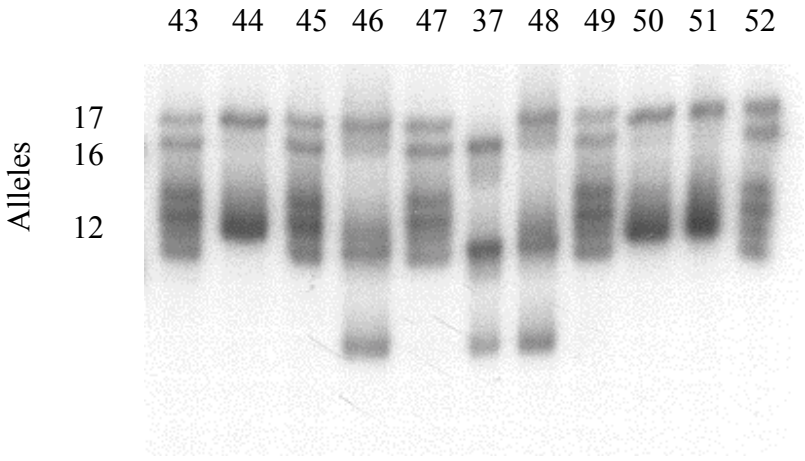

Figure 77. **Electrophoretic analysis of locus Aajμ5 amplification products 37 and 43-52.** The Spoonbill sample numbers are listed at the top of the figure and the allele designations are indicated on the left. This denaturing 4% polyacrylamide gel was electrophoresed at 50 W for 1 hour.

Table XII. **Combined genotypes (DNA profiles) of 51 Roseate Spoonbills**					
Fort Worth Zoo (22 Spoonbills)					
Spoonbill ID	Aajμ1	Aajμ2	Aajμ3	Aajμ4	Aajμ5
1	13,15	12,16	13,13	2,15	12,17
2	17,22	13,19	20,20	15,15	16,17
3	15,20	16,19	13,13	15,15	12,12
4	14,15	19,19	11,13	15,15	16,17
5	15,15	19,19	13,20	2,15	16,17
6	14,20	19,20	20,20	15,15	12,17
7	13,15	16,19	13,13	2,15	12,17
8	14,15	19,19	13,13	2,15	16,16
9	13,14	13,15	13,22	15,15	16,16
10	18,20	19,19	13,13	15,15	16,16
11	14,20	13,19	13,13	15,15	16,16
12	17,20	16,19	13,13	2,15	16,16
13	20,20	16,19	13,13	15,15	16,16
14	13,13	13,16	20,22	15,15	16,17
15	17,18	16,19	13,13	2,15	16,17
16	14,17	19,19	13,13	2,15	16,17
17	14,17	16,19	13,13	2,15	16,16
18	15,15	20,20	13,20	15,15	17,17
19	13,18	19,20	13,13	2,15	16,17
20	13,14	19,20	13,13	15,15	16,17
21	13,17	16,19	13,13	15,15	16,16
22	13,14	16,16	13,20	2,15	16,17
Dallas Zoo (20 Spoonbills)					
Spoonbill ID	Aajμ1	Aajμ2	Aajμ3	Aajμ4	Aajμ5
23	14,15	16,19	13,22	2,15	12,16
24	15,15	16,19	13,20	2,15	12,12
25	13,15	14,19	13,20	15,15	12,12
26	13,20	19,20	11,20	2,15	12,16
27	13,15	16,19	13,20	15,15	16,17

Table XII (continued). **Combined genotypes (DNA profiles) of 51 Roseate Spoonbills**					
Dallas Zoo (20 Spoonbills)					
Spoonbill ID	Aajμ1	Aajμ2	Aajμ3	Aajμ4	Aajμ5
28	13,15	16,20	13,20	2,15	16,17
29	13,15	19,19	13,20	2,15	16,17
30	13,13	13,13	13,20	2,15	17,17
31	15,21	14,20	13,20	2,15	16,17
32	14,21	18,19	13,20	15,15	17,17
33	13,21	14,20	13,20	15,15	16,17
34	15,21	14,20	13,22	2,15	16,17
35	13,13	15,20	20,20	2,15	12,16
36	13,20	19,19	11,13	15,15	12,12
37	15,20	19,20	13,20	2,15	12,16
38	14,15	19,19	13,20	15,15	17,17
39	13,13	19,20	13,20	2,15	12,12
40	13,14	19,19	13,20	15,15	16,17
41	21,21	14,20	13,22	15,15	16,17
42	13,13	14,19	11,13	15,15	12,12
Sedgwick County Zoo (9 Spoonbills)					
Spoonbill ID	Aajμ1	Aajμ2	Aajμ3	Aajμ4	Aajμ5
43	15,15	16,20	13,20	2,15	16,17
44	14,21	19,20	13,13	15,15	17,17
45	14,17	16,18	20,20	2,15	16,17
46	13,14	13,15	13,20	15,15	12,17
47	13,15	15,16	13,20	2,15	16,17
48	14,15	13,14	20,20	15,15	12,17
49	14,15	13,20	13,22	2,15	17,17
50	14,14	13,13	20,22	15,15	17,17
51	14,14	14,16	20,20	2,15	16,17

Table XIII. **Allele frequencies for Aajμ1**			
allele	N*	AF**	95% CI***
13	27	0.265	(0.179, 0.365)
14	22	0.216	(0.138, 0.310)
15	25	0.245	(0.164, 0.342)
16	0	0.000	
17	7	0.069	(0.028, 0.136)
18	3	0.029	(0.006, 0.084)
19	0	0.000	
20	10	0.098	(0.048, 0.173)
21	7	0.069	(0.028, 0.136)
22	1	0.010	(0.000, 0.053)
All	102	1.000	
homozygotes			13
heterozygotes			38
total samples			51
Observed Heterozygosity			0.75
Expected Heterozygosity			0.80
*N = number of alleles observed			
**AF = allele frequency			
***CI = 95% confidence interval for a binomial proportion (Zar, 1996, pp.524-525)			

Table XIV. **Allele frequencies for Aajμ2**			
allele*	N**	AF***	95% CI****
12	1	0.010	(0.000, 0.053)
13	11	0.108	(0.055, 0.185)
14	8	0.078	(0.034, 0.149)
15	4	0.039	(0.011, 0.097)
16	19	0.186	(0.117, 0.276)
17	0	0.000	
18	2	0.020	(0.002, 0.069)
19	40	0.392	(0.296, 0.495)
20	17	0.167	(0.101, 0.260)
All	102	1.00	
homozygotes			13
heterozygotes			38
total samples			51
Observed Heterozygosity			0.75
Expected Heterozygosity			0.76
*allele = allele designation based upon the largest repeat Note: alleles 14 and 15 binned; possibly are 14.2 and 15.2			
**N = number of alleles observed			
***AF = allele frequency			
****CI = 95% confidence interval for a binomial proportion (Zar, 1996, pp.524-525)			

Table XV. **Allele frequencies for Aajμ3**			
allele	N*	AF**	95% CI***
11	4	0.039	(0.011, 0.097)
12	0	0.000	
13	57	0.559	(0.456, 0.656)
14	0	0.000	
15	0	0.000	
16	0	0.000	
17	0	0.000	
18	0	0.000	
19	0	0.000	
20	34	0.333	(0.242, 0.432)
21	0	0.000	
22	7	0.069	(0.028, 0.136)
All	102	1.000	

homozygotes	21
heterozygotes	30
total samples	51
Observed Heterozygosity	0.59
Expected Heterozygosity	0.57

*AF = allele frequency

**N = number of alleles observed

***CI = 95% confidence interval for a binomial proportion (Zar, 1996, pp.524-525)

Table XVI. **Allele frequencies for Aajμ4**			
allele*	N**	AF***	95% CI****
2	26	0.255	(0.172, 0.354)
3	0	0.000	
4	0	0.000	
5	0	0.000	
6	0	0.000	
7	0	0.000	
8	0	0.000	
9	0	0.000	
10	0	0.000	
11	0	0.000	
12	0	0.000	
13	0	0.000	
14	0	0.000	
15	76	0.745	(0.646, 0.828)
All	102	1.000	

Note: This is a sex-linked microsatellite	
homozygotes (male)	25
heterozygotes (female)	26
total samples	51
Observed Heterozygosity	0.51
Expected Heterozygosity	0.380
*allele = allele designation based upon the largest repeat	
**AF = allele frequency	
***N = number of alleles observed	
****CI = 95% confidence interval for a binomial proportion (Zar, 1996, pp.524-525)	

Table XVII. **Allele frequencies for Aajμ5**			
allele*	N**	AF***	95% CI****
12	21	0.206	(0.132, 0.300)
13	0	0.000	
14	0	0.000	
15	0	0.000	
16	41	0.402	(0.303, 0.505)
17	40	0.392	(0.296, 0.495)
All	102	1.000	
homozygotes			21
heterozygotes			30
total samples			51
Observed Heterozygosity			0.59
Expected Heterozygosity			0.64
*allele = allele designation based upon the largest repeat			
**AF = allele frequency			
***N = number of alleles observed			
****CI = 95% confidence interval for a binomial proportion (Zar, 1996, pp.524-525)			

Spoonbill lineage analyses

An investigation of suspected Spoonbill families was undertaken to test the discriminatory power of these STR loci. The recorded mating pairs and presumed offspring of the Spoonbills were listed in the Specimen Reports and ancillary data provided by each zoo, which are summarized in Tables XVIII, XIX and XX. These suggested relationships were useful for testing the exclusionary power of these DNA-based tests.

If each of the alleles of the offspring were present in at least one of the parents, then the results were considered to be inclusive. However, if even 1 of the offspring's alleles was not accounted for within the profiles of the parents, then the results were considered to be exclusionary. A total of 25 suggested lineages were reported. Of these, 10 offspring were exclusions (5 excluded both dam and sire, with 3 dam and 2 sire single exclusions), indicated by "No" under "Possible offspring" (Table XXI). Each of these exclusions was investigated to determine if it was possible to identify parents.

The recorded lineages of 6 Spoonbills (#31, #34, #35, #43, #44 and #45) were investigated to help explain any possible Mendelian-based inheritance of a sex typing aberration (page 132). Since 4 independent tests (3 DNA based and 1 morphological) performed on Spoonbill #33 indicated that it was incorrectly sex typed during surgery, its misidentified sex type was considered to be independent of the female sex typing aberrations. However, the surgical report did state that the ovaries were very immature. The aberrant allele may have affected functional development of the male reproductive organs. The validity of this hypothesis is not supported well since two different sires from the wild population produced female offspring containing the aberrant allele.

Table XVIII. **Recorded lineages and mating pairs of Fort Worth Zoo Roseate Spoonbills**

*Sample #	Fort Worth Zoo ID	Recorded Dam x Sire [samples not acquired]	Source/Date of birth
1	950616	wild x wild	Choc., TX egg/June 8, 1995
2	950605	wild x wild	Choc., TX chick/June 6, 1995
3	950607	wild x wild	Choc., TX chick/June 6, 1995
4	950617	wild x wild	Choc., TX egg/June 8, 1995
5	950604	wild x wild	Choc., TX chick/June 6, 1995
6	950606	wild x wild	Choc., TX chick/June 6, 1995
7	950620	wild x wild	Choc., TX egg/June 9, 1995
8	950619	wild x wild	Choc., TX egg/June 8, 1995
9	950610	wild x wild	Choc., TX chick/June 6, 1995
10	O579	11 x 15	FW Zoo/June 24, 1981
11	O235	wild x wild	Rockport, TX/June 16, 1970
12	O584	([O241] x [O242]) or (11 x 15)**	FW Zoo/June 25, 1981
13	930501	10 x 12	FW Zoo/May 1, 1993
14	940574	wild x wild	Galv., TX egg/May 22, 1994
15	O240	wild x wild	Rockport, TX/June 17, 1976
16	O485	([O233] or [O234]) x ([O232] or #11)	FW Zoo/June 27, 1978
17	930576	21 x 16	FW Zoo/May 28, 1993
18	970610	[940559] x [930631]	FW Zoo/June 17, 1997
19	O1577	[0238] x 15	FW Zoo/June 8, 1989
20	940639	[0238] x 19	FW Zoo/June 17, 1994
21	O527	[0232] x 15	FW Zoo/July 14,1980
22	930633	wild x wild	Unknown wild/May, 1993

Choc. = Chocolate Bayou

Galv. = Galveston Bay

*Sample # assigned arbitrarily ** documented as 11 x 15, by Rebecca Linn's thesis (1993)

Table XIX. **Recorded lineages and mating pairs of Dallas Zoo Roseate Spoonbills**

*Sample #	Dallas ID	Sedgwick ID	Recorded Dam x Sire [ ] = sample not collected	Source/Date of Birth
23	823308		wild x wild	Rockport, TX/May 1982
24	823309		wild x wild	Rockport, TX/May 1982
25	823311		wild x wild	Rockport, TX/May 1982
26	833689		wild x wild	Nueces Bay, TX/May 1983
27	844071		[2189] x [3754]	Dallas Zoo/June 23, 1984
28	854407		wild x wild	W.Nueces Bay, TX/May 1985
29	854411		wild x wild	W.Nueces Bay, TX/May 1985
30	854414		wild x wild	W.Nueces Bay, TX/May 1985
31	928065	5442	(43 or 45) x 44	Sedgwick Co. Zoo/Aug. 6,1992
32	94C135	6756	[2670] x 46	Sedgwick Co. Zoo/July 28, 1994
33	95C136	7222	[2670] x 44	Sedgwick Co. Zoo/July 7, 1995
34	95C137	7224	[2670] x 44	Sedgwick Co. Zoo/July 8, 1995
35	96C139	7639	43 x 44	Sedgwick Co. Zoo/July 29, 1996
36	OOD401		29 x 32	Dallas Zoo/June 20, 2000
37	OOD402		29 x 32	Dallas Zoo/June 20, 2000
38	OOD404		28 x (33 or 34)	Dallas Zoo/June 20, 2000
39	OOD406		26 x 25	Dallas Zoo/June 20, 2000
40	OOD407		26 x 25	Dallas Zoo/June 20, 2000
41	OOD408		26 x 25	Dallas Zoo/June 20, 2000
42	OOD437		26 x 25	Dallas Zoo/July 6, 2000

*Sample numbers assigned arbitrarily

Table XX. **Recorded lineages and mating pairs of Sedgwick County Zoo Roseate Spoonbills**

*Sample #	Sedgwick Co. Zoo ID	Dallas Zoo ID	Recorded Dam x Sire [ ] = sample not collected	Source/Date of Birth
43	2668	864681	Dallas Zoo; unknown	Dallas Zoo/May 2, 1986
44	3454		wild x wild	wild/Louisiana/April, 1985
45	3471	886242	30 x [854412]	Dallas Zoo/June 13, 1988
46	3473	886232	[833685] x [833692]	Dallas Zoo/May 26, 1988
47	5422	928062	**[2670] x 46	Sedgwick Zoo/July 24, 1992
48	6745		**[2670] x 46	Sedgwick Zoo/July 25, 1994
49	7637	96C138	**[2670] x 46	Sedgwick Zoo/July 20, 1996
50	9619		Sedgwick Co. Zoo; unknown	Sedgwick Zoo/Feb. 17, 2001
51	9628		45 x 48	Sedgwick Zoo/Feb. 28, 2001

*Sample numbers arbitrarily assigned

**[2670] died March 3, 1998

Table XXI. **DNA profile analyses at 5 STR loci of recorded Spoonbill relationships**						
Shaded areas indicate observed problems						
Brackets, [ ], indicate a sample which was not acquired for this study						
A dash, -, indicates a reference sample						
Spoonbill #2670 alleles in parentheses, (), were deduced: see text						
Relationship	Possible	Aajμ1	Aajμ2	Aajμ3	Aajμ4	Aajμ5
	offspring					
Sire: #11	-	14,20	13,19	13,13	15,15	16,16
Dam: #15	-	17,18	16,19	13,13	2,15	16,17
Offspring: #10	Yes	18,20	19,19	13,13	15,15	16,16
*Offspring: #12	Yes	17,20	16,19	13,13	2,15	16,16
*Rebecca Linn's study indicated 11 x 15 instead of [0241 x 0242], which were not obtained						
Sire: #10	-	18,20	19,19	13,13	15,15	16,16
Dam: #12	-	17,20	16,19	13,13	2,15	16,16
Offspring: #13	Yes	20,20	16,19	13,13	15,15	16,16
Sire: [0232]	-					
Sire: #11	-	14,20	13,19	13,13	15,15	16,16
Dam: [0233]	-					
Dam: [0234]	-					
Offspring: #16	Yes	14,17	19,19	13,13	2,15	16,17
Sire: #21	-	13,17	16,19	13,13	15,15	16,16
Dam: #16	-	14,17	19,19	13,13	2,15	16,17
Offspring: #17	Yes	14,17	16,19	13,13	15,15	16,16
Sire: [0238]	-					
Dam: #15	-	17,18	16,19	13,13	2,15	16,17
Offspring: #19	Yes	13,18	19,20	13,13	2,15	16,17
Sire: [0238]	-					
Dam: #19	-	13,18	19,20	13,13	2,15	16,17
Offspring: #20	Yes	13,14	19,20	13,13	15,15	16,17

Table XXI (continued). **DNA profile analyses at 5 STR loci of recorded Spoonbill relationships**

Relationship	Possible offspring	Aajμ1	Aajμ2	Aajμ3	Aajμ4	Aajμ5
Sire: [0232]	-					
Dam: #15	-	17,18	16,19	13,13	2,15	16,17
Offspring: #21	Yes	13,17	16,19	13,13	15,15	16,16
Sire: #44	-	14,21	19,20	13,13	15,15	17,17
Dam: #43	-	15,15	16,20	13,20	2,15	16,17
Dam: #45	-	14,17	16,18	20,20	2,15	16,17
Offspring: #31	No for #45 &?	15,21	14,20	13,20	2,15	16,17
Sire: #46	-	13,14	13,15	13,20	15,15	12,17
Dam: [2670]	-	(13,15)	(14,20)	(20,22)	(2,15)	(16,17)
Offspring: #32	No	14,21	18,19	13,20	15,15	17,17
Sire: #44	-	14,21	19,20	13,13	15,15	17,17
Dam: [2670]	-	(13,15)	(14,20)	(20,22)	(2,15)	(16,17)
Offspring: #33	Yes	13,21	14,20	13,20	15,15	16,17
Offspring: #34	Yes	15,21	14,20	13,22	2,15	16,17
Sire: #44	-	14,21	19,20	13,13	15,15	17,17
Dam: #43	-	15,15	16,20	13,20	2,15	16,17
Offspring: #35	No	13,13	15,20	20,20	2,15	12,16
Sire: #32	-	14,21	18,19	13,20	15,15	17,17
Dam: #29	-	13,15	19,19	13,20	2,15	16,17
Offspring: #36	No	13,20	19,19	11,13	15,15	12,12
Offspring: #37	No	15,20	19,20	13,20	2,15	12,16
Sire: #33	-	13,21	14,20	13,20	15,15	16,17
Sire: #34	-	15,21	14,20	13,22	2,15	16,17
Dam: #28	-	13,15	16,20	13,20	2,15	16,17
Offspring: #38	No	14,15	19,19	13,20	15,15	17,17

Table XXI (continued). **DNA profile analyses at 5 STR loci of recorded Spoonbill relationships**						
Relationship	Possible	Aajμ1	Aajμ2	Aajμ3	Aajμ4	Aajμ5
	offspring					
Sire: #25	-	13,15	14,19	13,20	15,15	12,12
Dam: #26	-	13,20	19,20	11,20	2,15	12,16
Offspring: #39	Yes	13,13	19,20	13,20	2,15	12,12
Offspring: #40	No	13,14	19,19	13,20	15,15	16,17
Offspring: #41	No	21,21	14,20	13,22	15,15	16,17
Offspring: #42	Yes	13,13	14,19	11,13	15,15	12,12
Sire: [854412]	-					
Dam: #30	-	13,13	13,13	13,20	2,15	17,17
Offspring: #45	No	14,17	16,18	20,20	2,15	16,17
Sire: #46	-	13,14	13,15	13,20	15,15	12,17
Dam: [2670]	-	(13,15)	(14,20)	(20,22)	(2,15)	(16,17)
Offspring: #47	No	13,15	15,16	13,20	2,15	16,17
Offspring: #48	Yes	14,15	13,14	20,20	15,15	12,17
Offspring: #49	Yes	14,15	13,20	13,22	2,15	17,17
Sire: #48	-	14,15	13,14	20,20	15,15	12,17
Dam: #45	-	14,17	16,18	20,20	2,15	16,17
Offspring: #51	Yes	14,14	14,16	20,20	2,15	16,17

An analysis of the DNA profiles indicated that Spoonbill #31 could not have been the offspring of dam #45 (Table XXI). Additionally, one of the other possible parents, either #43 or #44 was excluded. If the mutant sex typing allele was Z-linked, the sire #44 must be the correct parent and #43 must not be the dam of #31. Spoonbill #46 had reportedly mated with #2670 in 1992, but must have mated with #43 as well (see below). This fact indicates that #2670 was present to mate with #44. Spoonbill #31 was likely the offspring of #2670 (alleles derived, see below) and #44, which were also the parents of #33 and #34.

Spoonbill #35 was suspected as the likely offspring of #43 and #44. However, an analysis of the DNA profiles indicated that neither #43 nor #44 could be a parent of #35 (Table XXI). The most likely parents of #35 were #2670 and #46. These birds were documented as producing offspring in 1992 (#47), 1994 (#48) and 1996 (#49). Spoonbill #35 hatched in 1996. An analysis of the DNA profiles from Spoonbills #47, #48 and #49, relative to their sire #46, indicated that dam #2670 provided all the necessary alleles to produce Spoonbill #35, except allele "13" from locus Aajμ1. However, an analysis of the DNA profiles from offspring #33 and #34, from the union of #44 and #2670, showed that #2670 must have contributed allele "13" to #33. The deduced alleles of #2670 are as follows: Aajμ1 (13,15), Aajμ2 (14,20), Aajμ3 (20,22), Aajμ4 (2,15) and Aajμ5 (16,17). This evidence supported the conjectures that the union of #2670 and #46 could have produced #35 and are the most likely the parents of #35.

The deduced alleles of #2670 helped to spotlight a problem with one of the Spoonbill lineages which was considered to be inclusive. Spoonbills #47, #48 and #49 were all considered to be offspring of #46 and #2670. However, the alleles of locus

Aajμ2 for these offspring were 15,16 (#47), 13,14 (#48), and 13,20 (#49). There are 5 different alleles present, but only 4 can be inherited from the parents. One of these Spoonbills was incorrectly included as an offspring. Alleles "13" and "14" of locus Aajμ2 must be inherited by Spoonbills #31, #32, #33, #34, #35, #48 and #49 from #2670. This means allele "16" must have been inherited from another dam. Spoonbill #43 was documented as a possible mate of #44 in 1992 (Table XIX), the same year that #47 hatched. Spoonbill #44 cannot be considered as a possible sire (it is excluded). However, #43 carries allele "16" at locus Aajμ2, and if it mated with #46 it could have produced #47.

Spoonbills #30 and #854412 were documented to be the parents of #45. An analysis of the DNA profiles indicated that #30 could not have been the dam of #45 (Table XXI). The evidence that Spoonbill #854412 was the sire of #45, besides the visual documentation, relies upon the hypothesis that the mutant allele is sex-linked (on the Z chromosome) and originated in the wild population. It is concluded that Spoonbill #45 most likely inherited the allele from #854412, or another Spoonbill brought in from the wild such as #833692, which was actively mating at the same time (in 1988 to produce #46).

Spoonbill #32 was documented to be the offspring of #46 and #2670. However, an analysis of the DNA profiles indicated that #46 was excluded as the sire of #32 (Table XXI). Interestingly, #44 had sired #31 in 1992 and in 1995 had mated with #2670 to produce 2 offspring (#33 and #34). All 3 offspring (#31, #33, and #34) carry the infrequent allele "21" from locus Aajμ1 (#44 is the only Spoonbill which carried allele "21" before the birth of #31, #33 and #34). Since #44 was present the year that #32 was

hatched, #44 cannot be excluded as the possible sire, especially considering that #32 has the rare "21" allele at the locus Aajμ1. A mating of #44 with #2670 (alleles derived above) must have produced #32.

Spoonbill #50 did not have documented parents. However, it was born about the same time as #51, in February 2001, which was documented as having the parents #48 and #45 (offspring listed as an inclusion, Table XXI). Spoonbill #50 contains the rare allele "22" from locus Aajμ3. Spoonbill #49 must have been the parent of #50 since it was the only mature Spoonbill containing allele "22" at the Sedgwick County Zoo. Spoonbill #48 was included as the possible sire. However, Spoonbill #46 does contain the proper alleles to have sired #50. The studbook records at the Sedgwick County Zoo would have to be reviewed to determine if #46 had access to #49. If both #46 and #48 were housed with #49, then the true sire of #50 cannot be determined until more polymorphic loci can be discovered and characterized.

The lineages from a group of 7 Spoonbills born during the summer of 2000 at the Dallas Zoo were investigated because 5 (Spoonbills #36, #37, #38, #40 and #41) of the 7 documented lineages were deemed exclusions (Table XXII). Six of the Spoonbills hatched about June 20th, 2000 and 1 Spoonbill (#42) hatched about July 6th, 2000. Since all of the parental Spoonbills were housed together in a large aviary, assignment of parentage by visual means was problematic. The DNA profiles from each of the 7 Spoonbills (#36 through #42) were compared with each of the potential parent's DNA profiles (3 sires and 4 dams) by alignment in a table (Table XXII), followed by analysis of them to deduce the necessary parentage. Four Spoonbills (#39, #40, #41 and #42) were the suspected offspring of sire #25 and dam #26. Two of these, #39 and #42, were

the offspring of #25 and #26. Two other Spoonbills, #36 and #37, were the offspring of #25 and #26. The other Spoonbill lineages were assigned as follows: Spoonbill #38 was the offspring of sire #32 and dam #29; Spoonbill #40 was the offspring of sire #32 and either dam #26 or #29; Spoonbill #41 was the offspring of sire #33 and dam #34.

Table XXII. **DNA profile analyses among 7 lineages**						
Possible Parents of the June 20th and July 6th, 2000 Offspring						
Sires		Aajμ1	Aajμ2	Aajμ3	Aajμ4	Aajμ5
25	alleles:	13,15	14,19	13,20	15,15	12,12
32	alleles:	14,21	18,19	13,20	15,15	17,17
33	alleles:	13,21	14,20	13,20	15,15	16,17
Dams						
26	alleles:	13,20	19,20	11,20	2,15	12,16
28	alleles:	13,15	16,20	13,20	2,15	16,17
29	alleles:	13,15	19,19	13,20	2,15	16,17
34	alleles:	15,21	14,20	13,22	2,15	16,17
Offspring, alleles, analyses and conclusions:						
Offspring		Aajμ1	Aajμ2	Aajμ3	Aajμ4	Aajμ5
#36	alleles:	13,20	19,19	11,13	15,15	12,12
	possible sire:	#25 or 33	#25 or 32	any	any	#25
	possible dam:	# 26	#26 or 29	any	any	#26
	conclusions:		**Sire must be #25. Dam must be #26.**			
#37	alleles:	15,20	19,20	13,20	2,15	12,16
	possible sire:	#25	Any	any	any	#25
	possible dam:	any	Any		any	any
	conclusions:		**Sire must be #25. Dam must be #26.**			
#38	alleles:	14,15	19,19	13,20	15,15	17,17
	possible sire:	#25 or 32	#25 or 32	any	any	#32 or 33
	possible dam:	any but #26	#26 or 29	any	any	any but #26
	conclusions:		**Sire must be #32. Dam must be #29.**			

Table XXII (continued). **DNA profile analyses among 7 lineages**						
#39	alleles:	13,13	19,20	13,20	2,15	12,12
	possible sire:	#25 or 33	any	any	any	#25
	possible dam:	any but #34	any	any	any	#26
	conclusions:		**Sire must be #25. Dam must be #26.**			
#40	alleles:	13,14	19,19	13,20	15,15	16,17
	possible sire:	any	#25 or 32	any	any	#32 or 33
	possible dam:	any but #34	#26 or 29	any	any	any
	conclusions:		**Sire must be #32. Dam must be either #26 or #29.**			
#41	alleles:	21,21	14,20	13,22	15,15	16,17
	possible sire:	#32 or 33	#25 or 33	any	any	#32 or 33
	possible dam:	#34	any but #29	any but #26	any	any
	conclusions:		**Sire must be #33. Dam must be #34.**			
#42	alleles:	13,13	14,19	11,13	15,15	12,12
	possible sire:	#25 or 33	any	any	any	#25
	possible dam:	any but #34	any but #28	any	any	#26
	conclusions:		**Sire must be #25. Dam must be #26.**			

Comparative statistics between the captive and the wild Spoonbills

During the course of investigating the Spoonbill lineages it became apparent that the Spoonbills from the Dallas and Sedgwick Zoos were reciprocally mated, which should have helped maintain a more genetically diverse population if performed without allowing inbreeding to occur. Family trees created by Jamie Kirk at the Sedgwick County Zoo (data not shown) in addition to the Specimen Report data provided by each zoo greatly assisted in the creation of Tables XIX and XX. The Specimen Report and studbook data provided by the Fort Worth Zoo assisted in the creation of Table XVIII. Based upon the suggested lineages, the degree of inbreeding within the captive populations was probably limited by the importation of wild birds (eggs or chicks) and by the exchange of Spoonbills with other zoos. In order to evaluate an assumption of limited inbreeding, statistical analyses of data from the wild and captive Spoonbill populations were performed.

Different groups of DNA profile data were combined as needed in order to calculate rates of observed heterozygosity (H_O) to be used in statistical analyses. The H_O rates (from each polymorphic locus) from a group of wild birds (21 total) were visually compared to the H_O rates (from each polymorphic locus) from a group of birds born in captivity (30 total). The H_O rates of Aajμ4 were not compared since this locus is linked to the Z and W chromosomes and thus does not produce a polymorphic profile independent of sex type. The H_O rates for the wild Spoonbill population (21) were as follows: Aajμ1 (0.810), Aajμ2 (0.714), Aajμ3 (0.571), Aajμ5 (0.619). The H_O rates for the captive Spoonbill population (30) were as follows: Aajμ1 (0.700), Aajμ2 (0.767), Aajμ3 (0.600), Aajμ5 (0.567). Only 2 of the 4 loci exhibited a decrease in H_O from the

wild population sample to the captive population sample, whereas the H_O rates at the other 2 loci increased in value. Since a discernable pattern was not obtained from these data, the criterion used to define each population was improved.

Another analysis of H_O rates was then performed on DNA profile data which were more distinctly representative of both the wild and captive populations. The DNA profiles of 15 Spoonbills from the wild population (#s1-9, #14, #22, #23, #24, #28 and #30) were grouped together because they had not produced any documented (and verifiable) offspring. The H_O rate of each locus was as follows: Aajμ1 (0.733), Aajμ2 (0.667), Aajμ3 (0.600), Aajμ5 (0.667). Then the DNA profiles of 8 Spoonbills (#13, #17, #41, #47, #48, #49, #50 and #51) were grouped together because they were documented to be second generation captive Spoonbills. The H_O rate of each locus was as follows: Aajμ1 (0.500), Aajμ2 (0.875), Aajμ3 (0.500), Aajμ5 (0.500). Three of the 4 loci showed decreased heterozygosity (increased homozygosity). When these H_O rates were visually compared to the rates of the previous Spoonbill groups (21 wild, 30 captive) there appeared to be considerable difference, specifically since the 3 decreased H_O rates were now each at an unusually low 0.5. These data appear to support the hypothesis that inbreeding of the captive population is increasing the overall homozygosity of alleles within the population. This hypothesis is biologically important since increased homozygosity is directly related to an organism's susceptibility to diseases, specifically any that are expressed (or developed) in the homozygous recessive.

A statistical analysis of these data was performed in an attempt to qualify the differences. The calculation was performed only on locus Aajμ1 data since the difference (0.733 versus 0.500) was the largest of all 4 comparisons, and if significance

was reached then the calculations could be performed on the other H_O rates. The ratios of heterozygotes to homozygotes at locus Aajμ1 (11:4 in the wild population and 4:4 in the captive population) were subjected to statistical analysis using the G test for contingency analysis (Beitinger, 1994; Zar, 1996). This analysis was more appropriate than the X^2 (Chi square) analysis since three of the values were relatively small (<5), which would have artificially inflated the X^2 value. The adjusted G statistic (G_{adj}) was calculated to be 1.14. This value was compared to a X^2 distribution table at 1 degree of freedom. It was concluded that heterozygosity is not significantly different between population types in Roseate Spoonbills [Log likelihood (G) contingency test with William's correction, $0.5>p>0.25$]. The heterozygosities at this locus may not have been significantly different because of two reasons. One reason may be because the sample size of 23 individuals was most likely too small to obtain the $\alpha = 0.05$ level of significance. The other reason significance was not obtained was that the H_O rates of the populations (wild and captive) were not measurably different. To obtain significance, the H_O rate in the captive population would have to be lower (probably less than 0.4) in the same captive population. It is preferable that such a low level of heterozygosity (high homozygosity) is never reached in any Spoonbill population. Because the H_O rates from Aajμ1 were not significantly different, the other 3 polymorphic loci were not statistically evaluated. Their differences in H_O rates should be even less significantly different.

Both the homozygosity rates at each locus and differences in allelic frequencies between sample populations are considered to be biologically important even when statistical significance cannot be obtained. An analysis of Spoonbill DNA profiles determined the frequency of multiple homozygous loci. Fifteen of the 51 Spoonbills (#3,

#5, #11, #12, #16, #17, #21, #24, #35, #36, #38, #39, #42, #44, and #51) contained 2 out of 4 loci (50%) which were homozygous. Six of the 51 Spoonbill samples (#8, #10, #13, #18, #30 and #50) had 3 out of 4 loci (75%) which were homozygous. These 21 Spoonbills are considered to be the most inbreed of all 51 Spoonbills. Each zoo should evaluate the use of these birds in future breeding.

One factor which can help evaluate the utility of each bird is the determination of valuable alleles within a population. Then a bird identified as carrying a less frequent allele (more valuable) can be selectively breed with other birds. Comparisons were made between the allelic frequencies from 2 populations of Spoonbill samples with the Fort Worth Zoo as one group (22 samples) and the Dallas and Sedgwick County Zoos as the other group (29) since their birds were reciprocally mated. It is difficult to estimate the importance of an infrequent allele, such as "22" from locus Aajμ1. Only 1 allele "22" was present in the Fort Worth Zoo, and was therefore only at 1% the total Spoonbill population (51). The presence of a unique allele was not expected to reach statistical significance, because of the small sample size, but the allele does represent biological importance. It is a unique identifier and it is an indicator of genetic diversity within the population. If the bird successfully breeds, allele "22" may be inherited by the next generation, which would help maintain genetic diversity in the future population. Each of the allele frequencies were compared and evaluated statistically by the use of goodness of fit tests (Fisher Exact test and G test).

The frequency of each allele at each locus within a population was compared to the corresponding allele frequency within the other population. Two of the alleles were not present in one population ("21" of Aajμ1 and "14" of Aajμ2) and one allele ("17" of

Aajμ1) was present 6 times in the Fort Worth Zoo population but only 1 time in the Dallas/Sedgwick County Zoos population. These 3 alleles were tested for goodness of fit by using the Fisher Exact test. All of the other comparisons of allele frequencies were performed using the G test. A total of eight alleles had significantly different frequencies between the two populations (Table XXIII). These data allow a population manager to more easily identify allelic differences between the two zoo populations so that underrepresented alleles could be selected for by controlled breeding.

Table XXIII. **Alleles with statistically significant differences in frequencies between two different Spoonbill populations**

Locus	Allele	(n =) in FW Zoo*	(n =) in D/S Zoo**	p value***
Aajμ1	17	6	1	P = 0.024
Aajμ1	20	7	3	0.01>p>0.005
Aajμ1	21	0	7	P = 0.016
Aajμ2	14	0	8	p = 0.00874
Aajμ3	13	33	24	P > 0.001
Aajμ3	20	8	26	0.005>p>0.001
Aajμ5	12	5	16	0.05>p>0.025
Aajμ5	16	25	16	0.005>p>0.001
*FW Zoo = Fort Worth Zoo (22 Spoonbills, n = 44)				
**D/S Zoo = Dallas/Sedgwick Co. Zoos (29 Spoonbills, n = 58)				
*** p = value from Fisher Exact test (Zar, 1996)				
*** # > p > # from G test (Zar, 1996)				

CHAPTER IV

CONCLUSIONS

The ability to identify specific individuals within a population using genetic profiles of their genomes is a powerful tool for the researcher as well as the wildlife manager. This ability to identify individuals, coupled with the ability to verify parentage relationships, allows a researcher to study the social and genetic complexities within small or large populations. One very important aspect of a species' social organization involves the interactions of males with females, specifically relative to the maintenance of genetic diversity. The genetic diversity (size of the gene pool) of a population is inversely related to the degree of inbreeding, which is either a matter of circumstance such as a founder effect after a population decline or isolation, or an attribute of the social structure within the species, such as a preference to mate with relatives. The production of both viable offspring as well as genetically diverse individuals greatly affects the survival of a species in today's world of decreasing habitats, habitat alterations, pollution, nesting area disturbances and various predatory pressures.

Roseate Spoonbills continue to survive as wild populations in wetlands such as bays, estuaries, tidal pools and mangrove swamps, even though the species was almost extirpated from the United States during the early 1900's. The current estimate of Spoonbills in the wild, which range from the Gulf of Mexico to the northern border of Argentina, is about 20,000 birds (Chris Brown, personal communication). The greatest concentrations of Spoonbills appear to be in Texas (about 3,000), Florida (2,500) and the Yucatan peninsula, with only small groups (10-100) throughout the South American

countries. Since Spoonbills have been listed as a "species of special concern" (Florida Fish and Wildlife Conservation Commission, 1997), studies to understand the social structures, to measure genetic diversity of the species, and to identify any specific habitats which should be protected, must be soon completed in order to ensure the future of the species.

The current understanding of Roseate Spoonbill social structure includes observations that the birds are not completely monogamous, nor do they always care for their own eggs (Chris Brown, personal communication). The Fort Worth Zoo has observed extra-pair copulations in the Spoonbill population and in other species, such as the Ibises. These facts make the captive populations of Spoonbills ideal models for developing a genetic foundation for the study of colonial breeding birds. The recorded pair bonding of Spoonbills from 3 zoos [Dallas (Texas), Fort Worth (Texas) and Sedgwick County (Kansas)] provided an excellent starting point from which to measure the extent of extra-pair copulations. The application of DNA-based tests on the presumptive parents and offspring allows us to identify the actual parental relationships genetically. The genetic data obtained can now be used to develop guidelines for the management of captive and wild populations.

Identification of polymophic loci

When unique DNA sequences or single locus probes are not available for use on the genome of a particular species, the identification of individuals from one generation to the next has usually involved the use of one or more multilocus probes. Two different multilocus probes, pV47-2 (Longmire *et al*., 1990) and $(CA)_n$, were used in our initial

efforts screen the Spoonbill genome for readily identifiable polymorphic patterns via the RFLP approach. The multilocus probe pV47-2 did show promising polymorphic profile patterns with *Bgl*II-digested DNA samples. However, the pV47-2 probe showed little or no polymorphic patterns with Spoonbill genomic DNA digested with other restriction endonucleases. The second multilocus probe, $(CA)_n$, was used on *Bgl*II-digested DNA and hybridized to many regions, but the overall pattern was not readily interpretable as polymorphic. It was thus quickly determined that an alternative approach was needed to identify polymorphic loci.

One such approach was to identify polymorphic loci within the MHC region of the Spoonbill genome. The MHC β chain genomic clone CCII-7-1 (Xu *et al.*, 1989) was acquired and used to probe *Bgl*II-digested Spoonbill DNA samples. The banding patterns obtained showed few, if any, signs of polymorphism. However, since the DNA sequences of many β-chain exons from the chicken genome had been determined (Kaufman *et al*., 1989), we used these sequences as templates in design of PCR primers to amplify Spoonbill β1-chain exons, which could then be used for single nucleotide polymorphism (SNP) analyses of this likely polymorphic gene. Five sets of PCR primers were used in attempts to amplify homologous regions of the Spoonbill genome, with limited success. Apparently this region of the genome is so polymorphic as to be very poorly conserved between chickens and Spoonbills. The PCR primers did not possess sufficient sequence homology to Spoonbill genomic DNA and thus the binding was too weak to allow specific amplification. Since the earlier RFLP analysis with CCII-7-1 did not work, and none of the predicted PCR products were obtained using the 5 primer sets, it was decided to use a β-chain probe to screen a library of Spoonbill genomic DNA with

the hope of identifying the clone encoding the homologous region. The large genomic clones could then be subjected to DNA sequence analyses to identify specific regions likely to be polymorphic. Spoonbill specific PCR primers could then be designed, either RFLP or PCR based, to evaluate the polymorphism directly.

A size-fractionated (9-23 kb) library (Lambda DASH®II, Stratagene™) of Spoonbill genomic DNA was created and probes were evaluated for potential use with it. Since the CCII-7-1 probe revealed little or no polymorphism in the RFLP patterns with Spoonbill genomic DNA digested with *Bgl*II, this probe was not used to screen the library. The 5A/5B amplified product from chicken DNA (205 bp), which bound to multiple locations (8 distinct bands) in the chicken genome but only 1 or 2 places on the Spoonbill genome, was used as a heterologous probe to screen the Spoonbill, human and chicken genomes (Fig. 13). Because the probe seemed to bind rather specifically to the Spoonbill genome, it was considered a promising tool for the extraction of an MHC region from a suitable genomic library.

Four positively hybridized clones were isolated from the size-fractionated library using the probe 5A/5B. The Spoonbill DNA from each of these clones was isolated and processed through multiple rounds of subcloning until the DNA sequence was determined for each hybridizing region. The Spoonbill DNA sequences in fact showed very little homology to the chicken genomic sequence used to probe the library or to other MHC sequences present in the GenBank database. Again, the domestic chicken genome appeared to be distinctly different than the Spoonbill genome in this region. The lack of homology between the obtained sequences of Spoonbill DNA and those within

the GenBank database suggest that considerable differences exist at the nucleotide level between the published MHC regions and the Spoonbill's MHC.

The initial hope for success with this approach was based upon the morphological classifications of birds (Wetmore, 1960), which did not identify a great evolutionary distance (3 of 22 Orders) between the Order Ciconiiformes (includes Spoonbills) and the Order Galliformes (includes the domestic chicken). However, an alternative biochemical classification of birds (Sibley and Alquist, 1990) identified a considerable distance (18 of 21 Orders) between the same Orders, and has even placed them in the most divergent types of Superorders and Parvclasses. It may therefore be postulated that the chicken and Spoonbill MHC regions are as disparately organized as the enzymatic genes. If so, the nucleotide sequences of the obtained clones may in fact be part of the Spoonbill MHC even though the homology to the chicken DNA sequences was not obvious. Alternatively, the lack of good sequence identity may have forced the hybridization stringency to an unacceptable low level of specificity.

A second set of primer, Ex2a and Ex2b, was designed to amplify β exonic regions of the chicken's MHC, but they also failed to amplify the predicted product from Spoonbill DNA. A third set of primers, TCRAC1 and TCRAC2, was therefore designed to amplify a constant region of a chicken T-cell receptor α (TCRα) chain gene (Gobel *et al.*, 1994). This region was expected to be more similar between chickens and Spoonbills than the MHC β-chain because of the conserved nature of the TCRα gene structure between birds and even mammals (Göbel *et al.*, 1994). At a minimum this would verify the utility of chicken-derived probes to isolate homologous Spoonbill clones. The amplified product from the Spoonbill DNA appeared to be the same size (approximately

1,900 bp) as that obtained from amplified chicken DNA. The nucleotide sequence of the 1,942 bp clone was determined from "shotgun" subclones and subsequently compared to the GenBank database using the BLAST® search tool (Altschul *et al*., 1997). Eight DNA regions of the Spoonbill clone showed high homology (83 to 97%) with corresponding regions of a chicken (*Gallus gallus*) clone containing a T-cell receptor alpha chain gene (GenBank accession #U83833). This result demonstrated that PCR primers could be designed using at least some chicken gene sequences as templates and that a homologous region could be amplified with these primers and cloned from the Spoonbill genome.

Many studies have been performed on the domestic chicken as part of federal and commercial effort to limit the sensitivity of the birds to disease while at the same time selectively breeding them to produce the best possible meat as food. The relative abundance of chicken nucleotide sequences makes their use as heterologous probes for other species common. However, it is now clear that this option should be exercised with considerable care. The evidence obtained from this study supports the very diverse biochemical (versus morphological) classification of bird orders within the class Aves. Whereas numerous probes may be useful for a variety of mammalian species because of the relatively small level of genetic variability throughout the class Mammalia, probes from one order of birds may not be especially useful in others because of the considerable evolutionary radiation of avian genome diversity over time. Further attempts to develop polymorphic RFLP or SNP loci were suspended at this point in favor of isolating STR loci as described below.

Isolation of STR loci

As our attempts to isolate polymorphic loci from the MHC region slowly progressed, it became evident from the literature that microsatellite loci were rapidly becoming the standard for DNA-based identification studies (Gibbs *et al*., 1997; Prodöhl *et al*., 1996). For example, the genome of the armadillo, *Dasypus novemcinctus*, was screened for the presence of microsatellite loci (Prodöhl *et al*., 1996). Seven of 124 positively hybridized clones proved to be polymorphic microsatellite loci, which were subsequently used in PCR-based identity tests. These results showed the utility of microsatellite loci, which should theoretically be found in almost any higher order eukaryote. Therefore, an attempt was initiated to isolate microsatellite loci from the Spoonbill genome. Although dinucleotide STR loci are the most common variety, these were generally avoided in our studies due to their recalcitrant nature in typing procedures related to their tendency to "stutter" during the amplification process. Our efforts concentrated on trinucleotide and tetranucleotide STRs such as those used for human DNA-based testing procedures.

Even though STR loci are numerous within the genomes of higher eukaryotes, not all genomes have equivalent amounts of STR loci. For example, the frequency of STR loci in avian species is considered to be at least 10 times less than that observed in mammals (Weber and May, 1989; Crooijmans *et al*., 1993; Gibbs *et al.*, 1997). Therefore, the use of an efficient approach for the isolation of STR loci was paramount. The large insert Spoonbill genomic library we created in the vector Lambda DASH®II was considered a possible candidate for screening with a synthetic microsatellite probe. However, the vast nature of the large DNA inserts (9-23 kbp), relative to the 50 to 100 bp

microsatellite regions which would be bound by the probe, could require the creation of many dozens of subclones, followed by hundreds of DNA sequence determinations in order to isolate STR loci. Since STRs are generally relatively short, less than 200 bp, they can be effectively cloned into small insert libraries while maintaining the flanking regions necessary for PCR amplification. We therefore produced several small libraries of Spoonbill DNA, including ones enriched for STRs.

An STR enrichment procedure was adapted from papers by Li *et al*. (1997) and Gardner *et al*. (1999) in order to specifically extract STR loci from the Roseate Spoonbill genome. The enrichment technique utilized the properties of streptavidin-coated magnetic beads coupled to biotin-labeled capture oligonucleotides containing STRs (Fig. 1) to hybridize with genomic fragments which contain microsatellite loci. The enrichment procedure enabled the capture of hundreds of STR loci. However, the majority of these STRs were deemed unsuitable for a variety of reasons discussed previously (see Results and Discussion). From the DNA sequences of these clones, 23 sets of primers were designed and 5 were ultimately characterized as amplifying polymorphic loci (Table XXIV), although one locus, Aajμ6, was not used to differentiate alleles in this study. The STR enrichment and capture procedure, outlined in Materials and Methods, reflects the final version of the protocol's evolution through many modifications imposed during the numerous isolation attempts. The final procedure was also utilized with only minor adjustments by Richa Kapil (personal communication) to capture multiple STR loci from the genome of the Caribbean Flamingo (*Phoenicopterus ruber ruber*). As of this time, 10 Flamingo primer sets have been designed and 4 have resulted in the amplification of polymorphic loci from the Flamingo genome.

Table XXIV. **List of primer sets designed to amplify STR loci from the Roseate Spoonbill genome**			
Original name of clone	*Assigned name	Page #s	Notes
pGMS6/7R	Aajμ1	143, 147	polymorphic
pGMS10R/00	Aajμ2	158, 163	polymorphic
pGMS3/81		167, 320	single band amplified
pGMS04/5		170, 321	single band amplified
pGMS04/7	Aajμ3	170, 173	polymorphic
pGMS04/10		170, 322	single band amplified
pGMS9-6		177, 323	4 bands amplified/non-polymorphic
pGMSSB12		187, 324	multiple bands/non-polymorphic
pGMSSB19		187, 325	multiple bands/non-polymorphic
pGMS14H		192, 326	single band amplified
pGMS23H		192, 327	single band amplified/gene associated
pGMS18b		195, 196	multiple bands/polymorphic at 450 bp?
pGMS3/24F	Aajμ6	200, 202	polymorphic/bands not distinct
pGMS3/31		206, 328	multiple bands/non-polymorphic
pGMS3/46		207, 329	multiple bands/non-polymorphic
pGMS3/47		207, 330	no bands amplified
pGMS3/40		209, 331	multiple bands/non-polymorphic
pGMS16/38		211, 332	multiple bands/non-polymorphic
pGMS16/4B		212, 333	multiple bands/non-polymorphic
pGMS16/12B		214, 334	no bands amplified
pGMS16/29B		214, 335	multiple bands/non-polymorphic
pGMS16/32C	Aajμ4	215, 216	polymorphic/sex linked
pGMS16/45B		218, 336	multiple bands/non-polymorphic
pGMSZapEco[B]1/1		233, 337	non-polymorphic
PGMSE[2]1/1		235, 338	multiple bands/polymorphic at 550 bp?
PGMSE[2]2/1		235, 339	multiple bands/non-polymorphic
pGMS[3]5	Aajμ5	237, 238	polymorphic
pGMS[B]4		239, 340	multiple bands/non-polymorphic
pGMS[2]3		239, 341	single band amplified
pGMS3Δ5		242, 342	multiple bands/non-polymorphic
PGMSE[4]1/2		243, 343	no bands amplified
pGMSZapEco[B]3/1		243, 344	multiple bands/non-polymorphic
	*sequence submitted to GenBank		

The efficiency of STR capture via the enrichment method was compared to a standard (non-enriched) STR isolation procedure utilizing 2 small-insert (400-800 bp) Spoonbill genomic DNA libraries which were screened with 4 STR oligonucleotides. A total of 25 positively hybridized plaques were purified and excised to produce pBK-CMV phagemid clones. The nucleotide sequences of each clone were determined using T3 and T7 primers. A variety of STR loci were obtained, including some containing anomalous sequences which did not encode distinct STR loci. However, almost one-half of the loci contained a specific STR with intact flanking regions. Nine loci were chosen for the design of PCR primers (Table XXIV) and one was subsequently identified as a polymorphic locus (Aajμ5). Future attempts at cloning STR loci with this method should further limit the library insert size to between 300 and 600 bp. The smaller clones will assist in determining the STR sequence without requiring sequence analysis of hundreds of additional base pairs per clone.

Both the enriched and standard methods of STR isolation have benefits as well as disadvantages. Both methods allowed for the isolation of polymorphic loci. Each type of isolation was very labor intensive, especially since the DNA sequences of the clones must be determined individually. One advantage of creating a standard small-insert DNA library is that it may be created in a variety of cloning vectors, such as pUC18, pBluescript™, M13 or ZAP™ Express, which many laboratories have as part of their normal inventory. A disadvantage of the enriched method of STR capture is the need for unique items, such as paramagnetic particles, a magnet, biotinylated oligonucleotides and adapter oligonucleotides. A number of the clones obtained also appeared to be chimeric clones created as artifacts of the procedure. However, the reduced screening required for

enriched libraries is an obvious advantage that makes this method the method of choice for most laboratories. Many different types of DNA clones were obtained, including numerous truncated clones containing only one flanking region for the STR locus. In the end, 6 polymorphic STR loci were obtained from a total of 32 primer sets designed and tested.

Characterization of alleles at polymorphic loci

The discovery of polymorphic loci within the genome of an organism is only the first step in developing and utilizing unique profiles for individuals. The alleles of each locus must then be characterized with regard to their frequency and distribution within a population. Loci with alleles occurring at high frequencies (0.8 to 0.99) have a substantially reduced utility as indicators of identity or in other studies requiring genetic diversity (Queller and Goodnight, 1989). For example, loci with 4 alleles each at a frequency of 0.25 are much more informative than those with one allele at 0.9 and three others at 0.03. At a frequency of 0.9, approximately 8 out of 10 samples will be homozygous for this allele, which would greatly limit differentiation of samples at this locus.

After each polymorphic locus was isolated from the Spoonbill genome, a series of amplifications and gel electrophoreses were performed in order to DNA type each available Spoonbill sample. The alleles produced from each locus were identified by their sizes relative to the original sequenced clone, which contained a specific number of repeats within the STR locus. For example, locus Aajμ3 encoded an STR with the motif GAT repeated 13 times. The size of the amplified product for allele "13" is 176 bp. All

other alleles were sized using this allele as a reference point. Since another amplified product of locus Aajμ3 was 170 bp, it is referred to as allele "11" because it is 2 repeats smaller than allele "13". Once the alleles of each locus were identified, allele numbers could be assigned to samples as they were amplified. An allelic profile was created for each Spoonbill and the collected profiles were compiled into allelic tables, a separate one for each locus (see Appendix I, Tables XXV-XXIX). All 51 DNA profiles were further combined into a single table to assist in the analysis of suspected relationships (Table XII, page 264).

The distribution of alleles at polymorphic STR loci can also be informative, especially when the alleles are not continuous. The evolution of STR/microsatellite allele diversity, through expansion and contraction, is hypothesized to have occurred by the phenomenon of strand slippage during DNA replication (Levinson and Gutman, 1987: Schlötterer and Tautz, 1992; Zhu *et al*., 2000). As polymorphic STR loci evolved one would commonly expect to see "bell shaped" distributions of alleles/frequencies. This is what is observed with the majority of the 13 human STR loci used for standard forensic and parentage testing. Widely disparate alleles (missing intermediate sizes) can be suggestive of a loss of allelic diversity such as would occur during a population bottleneck. Evidence of this phenomenon may be derived from the analysis of STR allelic data.

A normal distribution of alleles was not observed in many of the Spoonbill loci we studied (Tables XIII-XVII, pgs 266-270). For example, locus Aajμ2 appeared to have a tri-nodal distribution (alleles 13, 16 and 19) instead of a more continuous radiation of alleles from a single ancestral STR allele. Additionally, as seen at locus Aajμ3, the

frequencies of common alleles "13" and "20" were at 56% and 33%, respectively, whereas the adjacent alleles had a frequency of zero. The absence of intervening alleles (frequency = 0) was also observed at other loci. Although it could be that these other allele sizes were never observed or common in Spoonbills, such examples of allelic distribution could also have originated from the population bottleneck experienced by these birds. During the 30 years prior to 1920 the population of Roseate Spoonbills in Texas (the only known Spoonbills in the United States) consisted of only a few hundred individuals (Allen, 1942), with the majority of these failing to pair and breed. Thus very few Spoonbills (only 20 to 25 breeding pairs nesting in 3 colonies) passed on their alleles to the next generation. The genetic consequences of this population collapse may have been further exacerbated by the survivors being derived from isolated, potentially somewhat inbred, groups. The severe decrease in the number of breeding individuals greatly increases the probability that some of the alleles were lost while others progressed towards fixation. Since all 51 Spoonbills acquired for this study are descendants of the wild population within Texas and Louisiana, it would not be surprising if many alleles once present in the entire Spoonbill population were not observed in our population study. It will be of interest to analyze samples collected from Mexican or South American birds in the future.

Determination of the allelic profiles of individuals at each locus (Tables XIII-XVII, pgs 266-270) allowed us to calculate the observed number of heterozygotes (H_O). The expected heterozygosities (H_E) were then calculated from allele frequencies and compared to the H_O. Since most of the rates of H_O and H_E were similar (the rates of Aajμ4 differed the most), it does not appear that a significant number of null or invisible

alleles were missed. This would have lead to an increased level of observed homozygosity which was not seen. Since locus Aajμ4 is linked to homologous but non-recombining regions of the Z and W chromosomes, the H_O (0.51) differed somewhat from the H_E (0.38). This discrepancy indicated a non-random distribution of alleles and specifically that homozygous "2" is lethal. This is of course true, but the lethality is unrelated to the Aajμ4 locus. For example, a male bird has the alleles "15" and "15". A female bird has the alleles "2" and "15". Therefore, in a sample with an equal number of male and females, the ratio of allele "15" to allele "2" will always be 3:1, but the H_O will be 0.5 and the H_E will be 0.375. To summarize, the H_E value is less than the H_O value in locus Aajμ4 since the alleles assort by sex, and not by random distribution during gametogenesis. Only Z,Z and Z,W individuals emerge from the reproductive process.

The H_O rates could also be used in comparative studies of populations. However, the initial hope to use 4 sample populations (wild samples separated from the 3 zoo samples) in H_O analyses was not implemented since the sample sizes would be too small to gain statistical significance. Therefore, two sample populations were created (21 wild and 30 captive) from which the H_O rates were calculated. A distinct change in H_O rates was not observed between these populations (2 rates increased and 2 rates decreased only moderately), therefore two more sample populations were created (15 from the wild with no offspring, and 8 second generation captive). A statistically significant difference of H_O rates was not obtained with the second set of populations even though the H_O rates in 3 out of 4 polymorphic loci (Aajμ4 not included) dropped to 0.5 in the captive population. Significance was not obtained because there was not a large enough difference between the two H_O rates, and/or the sample size (23) was too small. This data

is biological important because of the decrease in H_O rates from the wild population to the second-generation captive population, which most likely indicates a decrease in genetic diversity even though these data cannot be supported by statistical significance.

Additional statistical analyses were performed on the differences of allelic frequencies between the Fort Worth Zoo population (22) and the combined Dallas and Sedgwick County Zoo population (29). These populations were chosen because of their relatively large sample sizes (to gain significance) and the fact that the Dallas and Sedgwick County Zoo Spoonbills were reciprocally mated, but were not exchanged with the Fort Worth Zoo Spoonbills. Eight alleles (3 from Aajμ1, 1 from Aajμ2, 2 from Aajμ3 and 2 from Aajμ5) were identified as having significant differences in their frequencies (Table XXIII). This information indicated that the proportion of alleles has changed between the captive populations. This is relevant because these populations should be maintained as part of genetically large but separate groups. Since these alleles have been identified as the most divergent between these populations, they can be used to determine which Spoonbills should be exchanged between zoos in order to maintain the most genetically balanced captive populations possible.

Because of the limited allelic diversity discovered during the evaluation of this sample population of 51 Spoonbills, the allelic data in the larger population of Spoonbills should be further explored. Large randomly mating populations which frequently exchange members with other populations would be expected to have similar and relatively "complete" complements of alleles. If the allelic frequencies differ significantly between wild populations, then the Spoonbill populations may now be effectively islands of inbreeding birds as a result of their fragmented habitats. Since

Spoonbills in Texas and Louisiana reportedly migrate to Mexican rookeries (Allen, 1942), the allelic data from the Yucatan samples might not be expected to differ significantly from this sample population. However, the Spoonbills in Florida reportedly migrate to Cuba and the Bahamas, usually not along the Gulf of Mexico to Louisiana and Texas. Therefore, a survey of the allelic frequencies in these birds could yield data considerably different from that obtained from this sample population. The presence of additional STR alleles not detected (zero frequency) in this study would of course also suggest the presence of additional alleles at more important quantitative trait loci (QTL) and thus demonstrate the importance of introducing members of these populations into the Texas breeding programs. Additionally, the Spoonbills in Argentina and Brazil reportedly do not migrate any considerable distance. Therefore, their allelic data may also provide evidence of some "missing alleles" relative to the characterized STR loci from this Texas sample population and again show the importance of reconnecting habitats at least artificially by transfer of individuals.

Sex typing of Roseate Spoonbills

One of the most important tools used to manage a captive species is the knowledge of each animal's sex type. Once the sex is known, the sexual balance in the population can be maintained and the behavioral characteristics unique to each sex can be studied. A variety of bird species, including the Roseate Spoonbill and Flamingos, exhibit a lack of obvious external sexually dimorphic characteristics which can be determined from a distance. Under these circumstances the sex of each bird may only be obtained through direct methods, such as endoscopy (a surgical procedure to determine

sex), karyotyping (sex chromosomes identified), or from DNA analysis. DNA can be extracted from a variety of sources, most commonly from a feather pulled from the chest or from a very small sample of blood (5 µl).

A DNA-based sex test specifically for Roseate Spoonbills has not been published. However, in 1998 Griffiths *et al*. published a paper which described the design of 2 primers, P2 and P8 (Table III), which could be used to sex type most birds throughout the class Aves, excluding ratites (the early branch of birds including ostriches, emus, cassowaries, and a few other species). Fifty-one different samples of Roseate Spoonbill DNA were extracted from whole blood during the course of this study. A zoo Specimen Report was acquired for each sample and this provided the recorded sex type of at least some of the Spoonbills. These samples were chosen as positive controls to test the utility of the P2 and P8 PCR primers for distinguishing Spoonbill sex types. Blood samples from 9 Caribbean (American) Flamingos (*Phoenicopterus ruber ruber*) were also generously provided by the Fort Worth Zoo in order that DNA-based sex testing could be performed on them as well.

Both the Spoonbill and Flamingo DNA samples were amplified with P2 and P8 primers, yielding products of approximately 400 bp in each case. Further analyses of the amplified samples by 7.5% (37.5:1) polyacrylamide gel electrophoresis showed the products to be either 390 or 396 bp, corresponding to the Z and W chromosome products, respectively. The DNA sequences were determined from the amplified Z chromosome products and submitted to the GenBank database: 302 bp from the Roseate Spoonbill, accession #AF440750, and 304 bp from the Caribbean (American) Flamingo, accession #AF440751. All of the Spoonbill (22) and Flamingo (9) DNA samples from the Fort

Worth Zoo amplified the expected single band to indicate males (ZZ) or 2 bands to indicate females (ZW). Analyses of the Dallas Zoo (20) Spoonbill DNA samples revealed a problematic result. Three different DNA samples, these from birds #31, #33 and #34, were amplified with the P2 and P8 primers. After gel electrophoresis and staining with ethidium bromide, the products from each of these bird samples appeared to be a single band indicative of a male, instead of the 2 bands indicative of a female as they were reported to be. The primers P2 and P8 appeared to have lost their ability to differentiate sex types in at least some Spoonbills.

Shortly after the study characterizing the P2 and P8 primers was published, a second set of universal avian sex typing primers, 2550F and 2718R (Table III), were isolated (Fridolfsson and Ellegren, 1999). Even though these primers had been designed to amplify a portion of the same sex-linked gene (Chromo-Helicase-DNA (CHD) binding protein gene), the two amplified products of Spoonbill (and Flamingo) Z and W chromosomes were much more distinct. The primers amplified a 700 bp product from the Z chromosomes of male birds (Spoonbills and Flamingos). When the primers were used to amplify the DNA from female Flamingos, 2 bands were produced, one of 700 bp (Z chromosome) and one of 450 bp (W chromosome). However, only a single product was amplified from female Spoonbill DNA, a 450 bp fragment (W chromosome). This apparent W chromosome homozygosity is not an uncommon occurrence with many avian species when using this primer set. A possible explanation for the absence of an amplified Z chromosome product (700 bp) from the DNA of female Spoonbills has been put forward, with it resulting from a preferential amplification of the 450 bp product (Fridolfsson and Ellegren, 1999). Thus, a 700 bp fragment is amplified from Flamingo

males and two fragments, of 700 bp and 450 bp, are amplified from Flamingo females. In contrast, a 700 bp fragment is amplified from Spoonbill males while a single fragment of 450 bp is amplified from Spoonbill females. Although this latter case in a sense represents a failure to amplify one product, the sexes are still distinct with respect to the results, and so the utility of the test is unaffected.

Amplifications with the 2550F/2718R primer set of DNA from Spoonbills #31, #34 and #45 yielded interesting results. The amplified products of these samples were single bands at 450 bp, indicative of females. These results confirmed the sex types of the Dallas and Sedgwick County Zoos (Tables V and VI). However, the sex type of each of these appeared to be male when amplified with the P2/P8 primer set. From these discrepancies it was determined that the P2/P8 primer set was not functioning properly to amplify the sex chromosomes in all individuals of this species. Therefore, this primer set is unreliable with Spoonbills and the results demonstrate the importance of additional testing when not using species-specific primer sets.

During the course of capturing and characterizing STR loci a Z/W-linked STR locus exhibiting a sex-specific dimorphism in Spoonbills was in fact discovered. The primer set, Aajμ4F and Aajμ4R, was designed from locus Aajμ4 (Appendix I, Table XXVIII, and clone 16/32C, page 215) to amplify a 200 bp product from Spoonbill genomic DNA. Amplification of male Spoonbill DNA yielded a 200 bp fragment. Amplification of female Spoonbill DNA yielded 2 fragments: 200 bp and 161 bp. DNA from each of the 51 Spoonbill samples was amplified to determine the sex types (Tables IV, V and VI).

Since a species-specific sex-typing test was now available for the Roseate Spoonbill, the sex-typing results of #33 were further investigated. The primer set, Aajμ4F and Aajμ4R, amplified the DNA of Spoonbill #33 to yield one fragment, indicative of a male. Three DNA-based tests now indicated that #33 is a male. These results contrasted with the results of the surgical sexing procedure which indicated that #33 was female. One other independent test was utilized to ascertain the sex of #33. This test relied upon the measurement of a recently noticed sexual dimorphic characteristic. The length of a Spoonbill's bill has been associated with sex type (Jamie Kirk, personal communication). An investigation of this phenomenon led to the hypothesis that adult Spoonbills with a 16.2 cm bill or shorter are female, whereas males possess bills in the range of 16.5-18.2 cm (Laurie McGivern, personal communication). The bill length of Spoonbill #33 is 17.0 cm, which is comfortably within the range expected from a male Spoonbill. Therefore, it was ultimately concluded that the surgical procedure to determine the sex type of Spoonbill #33 was performed incorrectly.

The occurrence of the 3 female Spoonbills (#31, #34 and #45) with an aberrant CHD region, apparently on the Z chromosome, did not allow the P2/P8 differential amplification of 2 discernable bands. Perhaps the primer set could still be used to differentiate sex type by performing restriction endonuclease digestions (experiment not tested) on the amplified products before agarose gel electrophoresis in an attempt to visualize differences between male and female samples (Griffiths *et al*., 1998). However, this work is not necessary since 3 additional independent and accurate tests (two DNA based and one morphological) have now been characterized by this study. Identification of the founder Spoonbill harboring the mutated CHD region appears to have little

importance at this time. However, if an increased or reduced number of viable offspring is observed with these Spoonbills (#31, #33, #34, #44 and #45), some study of the mutated gene or region may prove to be beneficial to the species as a whole. At the very least, Spoonbill studbook managers can track these individuals in an attempt to evaluate their utility to the captive and wild populations.

Suspected familial relationships

Analyses of the studbook data (Tables XVIII, XIX and XX) assisted in identifying adult Spoonbills as potential parents. Each of the familial relationships recorded by collaborating zoos (Table XXI) was investigated by DNA profiling individual family members. Ten of the 25 (40%) were determined to be in error, with one or both of the recorded parents not having an allele present in the offspring. Discrepancies between suspected (by observation) and actual parentage were not unexpected since extra-pair copulations had been reported in captive Spoonbill populations (Chris Brown, personal communication). However, the 40% rate of misidentification of parental relationships by the zoos was considered to be quite high making studbook data of limited value in choosing prospective mating pairs.

Some of the corrected familial relationships assisted in the clarification of a problem identified during the sex typing of some individuals. The problem described earlier originated with the use of the P2/P8 primer set to amplify Spoonbill DNA samples. Three female Spoonbills were incorrectly sexed by the use of this primer set because the Z chromosome was amplified aberrantly resulting in only 1 visible fragment (which scored as male). The lineages of these Spoonbills were investigated in an attempt

to determine the founder of the aberrant typing phenomenon. The suggested lineages provided by the zoos were at best confusing, since the problem did not seem to follow a Mendelian pattern of inheritance. It was not until the DNA profiles of each Spoonbill became available that the lineages of Spoonbills could be determined properly and the carriers of the aberrant allele identified. Based upon the results of the DNA-based parentage analysis a simple Mendelian pattern of inheritance was determined for this phenomenon. This in a sense provided an internal validation of the DNA-based parentage determinations.

This is the beginning of a test to identify potentially deleterious alleles and trying to eliminate them from breeding populations in zoos. This test may be considered rudimentary, but in fact we have a locus which corresponds with a potential problem for zoo keepers, the ability to sex, which is in a gene. However, we don't know which allele may be superior under which conditions. Future breeding of the potential aberrant allele carriers (#33, #44 and the sire of #45) should be followed and their breeding success rates analyzed for either increases or decreases in fecundity, even though the mutation within the CHD gene may have no effect at all on the viability of future generations. However, the fact that #33 was identified as having very immature ovaries, when it was actually a male, is an interesting physical attribute which may very well have biological importance, depending upon the other physical attributes required for the reproductive success and survival of a normal male.

Concluding remarks

The colonial nesting behavior and the documented relationships of the Roseate Spoonbill made it an obvious choice as a paradigm for the development of a DNA-based identity and parentage test. The Regional Collection Plan & Three Year Action Plan of the American Zoo & Aquarium (AZA) Association, as of January 2001, reported the presence of 509 Roseate Spoonbills in 55 zoos, making it the most numerous Spoonbill in the AZA collections, compared to 87 individuals from two other species combined (in the wild there are 6 different species, representing 3 different genera). The Three Year Action Plan suggests the "management of reproduction, prevention of over-population and maintenance of the long-term demographic and genetic viability" within the species' entire captive population. By utilizing the PCR-based identity tests, samples from the AZA population and from the wild can be characterized and the data used to properly manage allelic diversity within the captive populations.

Although the choice of Roseate Spoonbills as a model system for study led to many unforeseen obstacles, it can also be considered fortunate in other ways. A fortunate aspect was the obvious need for a workable Spoonbill identity/parentage test. However, the difficulty of isolating polymorphic loci slowed the development of the test, and this was probably related to a more limited level of polymorphism in the Spoonbill genome. Of the 32 sets of PCR primers designed to amplify STR loci 6 amplified polymorphic loci, 6 produced a specific single band and 16 produced unique patterns of multiple bands (bound to multiple loci but still no evidence of polymorphism). Comparisons to other species (including Flamingo loci studied in our laboratory, see below) suggest that this level of polymorphism is rather low. Another positive aspect was the availability of

recorded family relationships which were used to characterize the discriminatory power of the tests. Since the relationships of many other bird species are not as well documented, the initial characterization of family lineages would have been more difficult if not impossible.

One of the more numerous colonial nesting birds is the Caribbean (American) Flamingo (*Phoenicopterus ruber ruber*). Its wild population is estimated to be in excess of 100,000 with multiple large colony breeding sites (Chris Brown, personal communication). The STR loci captured from its genome produced highly polymorphic patterns (Richa Kapil, personal communication). A near normal distribution of alleles was produced from the each of these loci and more alleles per locus were discovered relative to the Spoonbill genomic DNA capture. These results were expected since the DNA samples were obtained from a colony of 20,000 (blood samples obtained from juvenile birds), which has not undergone a population bottleneck and therefore should have had a considerable number of randomly mating breeding pairs throughout its existence. Development of a Flamingo DNA test first would have been useful to some researchers, but Spoonbills have been successfully bred in captivity for decades and have a reduced wild population, thus greater need for population management.

Differentiation of parentage in reasonably small populations such as found in zoos is a very important issue. The perceived future problem is that there is already a substantial drop in allelic diversity after one generation in captivity, which can be extrapolated to create problems to future generations. In order to better control the inheritance of alleles (and therefore genetic diversity), it may be necessary for certain sires to have limited access to dams. This would increase the chances for

underrepresented alleles to be maintained in the populations. More polymorphic loci could be isolated in order to differentiate individuals within some of the most homozygous (less heterozygous) populations in the future. However, this would not address the loss of genetic diversity within these populations. Properly exchanging birds between zoos and supplementing the zoo collections from properly chosen wild populations, only when absolutely necessary, could maintain the diversity.

The availability of a DNA typing test for Roseate Spoonbills may also prove to be genetically useful in typing the other five species of Spoonbills, although this possibility has not been verified to date. If these STR loci amplify the same regions within the endangered Black-faced Spoonbill (*Platalea leucorodia*) they could be used to monitor the allelic diversity within the population. Future work can also be done to evaluate the potential utility of the isolated STR loci in the family Threskiornithidae (Ibises and Spoonbills) and possibly the order Ciconiiformes, which includes herons, storks, flamingos, ibises, Spoonbills, as well as a number of other avian genera.

APPENDIX A

Table XXV. **Locus Aajμ1 core information**

GenBank accession #AF469474

Original clone ID: 6/7R (6/30/99) Repeat motif: (GAT)15

Primer Aajμ1F: GATCACCACCATCTTAAATGATAA

Primer Aajμ1R: CTTCTGTTTGCCTCACATGG

Annealing temperature: 55°C

Amplified product size for allele “15”: 163 bp

Fort Worth Zoo			Dallas Zoo			Sedgwick Co. Zoo	
Spoonbill ID	Alleles		Spoonbill ID	Alleles		Spoonbill ID	Alleles
1	13,15		23	14,15		43	15,15
2	17,22		24	15,15		44	14,21
3	15,20		25	13,15		45	14,17
4	14,15		26	13,20		46	13,14
5	15,15		27	13,15		47	13,15
6	14,20		28	13,15		48	14,15
7	13,15		29	13,15		49	14,15
8	14,15		30	13,13		50	14,14
9	13,14		31	15,21		51	14,14
10	18,20		32	14,21			
11	14,20		33	13,21			
12	17,20		34	15,21			
13	20,20		35	13,13			
14	13,13		36	13,20			
15	17,18		37	15,20			
16	14,17		38	14,15			
17	14,17		39	13,13			
18	15,15		40	13,14			
19	13,18		41	21,21			
20	13,14		42	13,13			
21	13,17						
22	13,14						

Table XXVI. **Locus Aajμ2 core information**							
GenBank accession #AF469475							
Original clone ID: 10F/00 (2/25/00) Repeat motif: (AACT)13TA(CTTT)6CT(CTTT)3							
Primer Aajμ2F: CTTGATGCAAAGGAAACATCC							
Primer Aajμ2R: GAGGTGCTTCCAGTTTCCTG							
Annealing temperature: 55°C							
Amplified product size for allele “13”: 217 bp							
Note: alleles 14 and 15 were binned: each appeared to be 1 or 2 nucleotides larger than expected							
Fort Worth	Zoo		Dallas	Zoo		Sedgwick Co.	Zoo
Spoonbill ID	Alleles		Spoonbill ID	Alleles		Spoonbill ID	Alleles
1	12,16		23	16,19		43	16,20
2	13,19		24	16,19		44	19,20
3	16,19		25	14,19		45	16,18
4	19,19		26	19,20		46	13,15
5	19,19		27	16,19		47	15,16
6	19,20		28	16,20		48	13,14
7	16,19		29	19,19		49	13,20
8	19,19		30	13,13		50	13,13
9	13,15		31	14,20		51	14,16
10	19,19		32	18,19			
11	13,19		33	14,20			
12	16,19		34	14,20			
13	16,19		35	15,20			
14	13,16		36	19,19			
15	16,19		37	19,20			
16	19,19		38	19,19			
17	16,19		39	19,20			
18	20,20		40	19,19			
19	19,20		41	14,20			
20	19,20		42	14,19			
21	16,19						
22	16,16						

Table XXVII. **Locus Aajμ3 core information**

GenBank accession #AF469476

Original clone ID: P04/7AB (4/30/00) Repeat motif: (GAT)13

Primer Aajμ3F: CCCATGGCCACATTATAAACTT

Primer Aajμ3R: GCTCTGGAGTAACTTGCTGGA

Annealing temperature: 55°C

Amplified product size of allele "13": 176 bp

Fort Worth Zoo			Dallas Zoo			Sedgwick Co. Zoo	
Spoonbill ID	Alleles		Spoonbill ID	Alleles		Spoonbill ID	Alleles
1	13,13		23	13,22		43	13,20
2	20,20		24	13,20		44	13,13
3	13,13		25	13,20		45	20,20
4	11,13		26	11,20		46	13,20
5	13,20		27	13,20		47	13,20
6	20,20		28	13,20		48	20,20
7	13,13		29	13,20		49	13,22
8	13,13		30	13,20		50	20,22
9	13,22		31	13,20		51	20,20
10	13,13		32	13,20			
11	13,13		33	13,20			
12	13,13		34	13,22			
13	13,13		35	20,20			
14	20,22		36	11,13			
15	13,13		37	13,20			
16	13,13		38	13,20			
17	13,13		39	13,20			
18	13,20		40	13,20			
19	13,13		41	13,22			
20	13,13		42	11,13			
21	13,13						
22	13,20						

Table XXVIII. **Locus Aajμ4 core information**							
GenBank accession #AF469477							
Original clone ID: 16/32C (6/26/01) Repeat motif: (GAT)2T(GAT)15							
Note: Sex specific							
Primer Aajμ4F: CCCTCCCAAGTGGTTACAGA							
Primer Aajμ4R: CTTGCATGTTCCAAATGGTG							
Annealing temperature: 58°C							
Amplified product size for allele "15" (Z chromosome): 200 bp							
Amplified product size for allele "2" (W chromosome): 161 bp							
Fort Worth	Zoo		Dallas	Zoo		Sedgwick Co.	Zoo
Spoonbill ID	Alleles		Spoonbill ID	Alleles		Spoonbill ID	Alleles
1	2,15		23	2,15		43	2,15
2	15,15		24	2,15		44	15,15
3	15,15		25	15,15		45	2,15
4	15,15		26	2,15		46	15,15
5	2,15		27	15,15		47	2,15
6	15,15		28	2,15		48	15,15
7	2,15		29	2,15		49	2,15
8	2,15		30	2,15		50	15,15
9	15,15		31	2,15		51	2,15
10	15,15		32	15,15			
11	15,15		33	15,15			
12	2,15		34	2,15			
13	15,15		35	2,15			
14	15,15		36	15,15			
15	2,15		37	2,15			
16	2,15		38	15,15			
17	2,15		39	2,15			
18	15,15		40	15,15			
19	2,15		41	15,15			
20	15,15		42	15,15			
21	15,15						
22	2,15						

Table XXIX. **Locus Aajμ5 core information**

GenBank accession #AF469478

Original clone ID: 3s5AB(10/29/01) Repeat motif: (GAT)16(TAT)4

Primer Aaj5F: GGCTGAACACTGTTGTGCTCT

Primer Aaj5R: GAACCAAGCCTCCCTGAATA

Annealing temperature: 58°C

Amplified product size for allele "16": 200 bp

Fort Worth Zoo			Dallas Zoo			Sedgwick Co. Zoo	
Spoonbill ID	Alleles		Spoonbill ID	Alleles		Spoonbill ID	Alleles
1	12,17		23	12,16		43	16,17
2	16,17		24	12,12		44	17,17
3	12,12		25	12,12		45	16,17
4	16,17		26	12,16		46	12,17
5	16,17		27	16,17		47	16,17
6	12,17		28	16,17		48	12,17
7	12,17		29	16,17		49	17,17
8	16,16		30	17,17		50	17,17
9	16,16		31	16,17		51	16,17
10	16,16		32	17,17			
11	16,16		33	16,17			
12	16,16		34	16,17			
13	16,16		35	12,16			
14	16,17		36	12,12			
15	16,17		37	12,16			
16	16,17		38	17,17			
17	16,16		39	12,12			
18	17,17		40	16,17			
19	16,17		41	16,17			
20	16,17		42	12,12			
21	16,16						
22	16,17						

APPENDIX B

Nucleotide sequence of clone pGMS3/81. See pages 167 and 296 for additional data.

```
10         20         30         40         50         60
GCAAAACTTG GGACAAAAAA ATAACCCCAT GCATTTTGGG GGGGAAAAGA GAAGGAACCC

        70         80         90        100        110        120
TGTGAGGTTT CCCCCATCTG TCTACATGCT CTGTTTGAAC ACTGACAGGA GAAAGAGCAT

       130        140        150        160        170        180
CCTCTGTGCT TCCATCCATC CTCCATCCAT CCATCCCTCC ATCCATCCAT CCGTCCGTCC

       190        200        210        220        230        240
GTCCAGAGTC TGAACATGTC AACATATCAG GCTCTGCAAG CCTGGAGAAT AGAACCTATT

       250        260        270        280        290        300
AGTTTATTTT AAATAGGCTT TTGCTTGGAA AA........ .......... ..........
```

Nucleotide sequence of clone pGMS04/5. See pages 170 and 296 for additional data.

```
10         20         30         40         50         60
AATAAAAATT AAAAAAAACA CAAAAATTAA AAAAAAAATT AAAATCACAA CTCCCGGGCT

        70         80         90        100        110        120
CGGAACTAAC TTCGAGATTT TTTTTTTTTT AAAGAAAACA AGACTTTTTT TTAATCATCA

       130        140        150        160        170        180
TCATCAGCAT CATCATCATC ATCACCGGCG TCGCGTCGCT CCAGCTCCGC CGCTGCCTCG

       190        200        210        220        230        240
CCGCCCGCCC TGGCCGCCGC CGCCCCCGCT CAGTACGTGA ACACCAGGTC GGAGAAGTTC

       250        260        270        280        290        300
GCCT...... .......... .......... .......... .......... ..........
```

Nucleotide sequence of clone pGMS04/10. See pages 170 and 296 for additional data.

```
10         20         30         40         50         60
GATCATCAAG TCCAACCGTT AACCCAGCAC TGCCAAGTCC ATCTATACCA TGTCTTCAAG

        70         80         90        100        110        120
CACCGTATCT ATCAGTTCTT TAAATACCTC CAGGGATGGA GACTCAACCA CTTCCCTGGG

       130        140        150        160        170        180
CAGCCTGTGC CAGTGCCTGA CCACCCTCTT GGTGAAGAAG TTTTTCCTAA TATCCAACCT

       190        200        210        220        230        240
AAACCTGCCC TGGCGCACGT TGAGGCCGTT TCCTCTTGTC CTATGACTCG TTCCTGGTGA

       250        260        270        280        290        300
GAAGAGACCG ACCCCCACCT CGCTCCAGCC TCCTTTCAGG TAGCTGTAGA GAGCAATAAG

       310        320        330        340        350        360
GCTCCCCTCA GATCCTCCTC TTCTCCAGGC TGAACACCCC CAGCTCCCTC AGCCGCTCCT

       370        380        390        400        410        420
CACAAGACTT GAATGGACAG GGAAGGATGG ATGGGAAAGG ATGGATGCAT GGATGGATGG

       430        440        450        460        470        480
ATGGATGGAG AAGAATGGAC AGGAAAGGAT GGATGGGGAA GGATGGATGG ATGGATGGAT

       490        500        510        520        530        540
GGATAAATAG ATGGATGGAT AGAGATGGAT GGGGATGAAT GGATGGGGAA GGGTAAGGAA

       550        560        570        580        590        600
CCCACCTAAG AAACCCATGG TGGCAGCTCT GCCATCTGAA GGGCGAGCTG AGCCCACCCC

       610        620        630        640        650        660
TCATATCCCT GTTGGCTCGG GGATC..... .......... .......... ..........
```

Nucleotide sequence of clone pGMS9-6. See pages 177 and 296 for additional data.

```
10         20         30         40         50         60
GATCAGAAAG AAGGGTGTGC CTCACTGATG AGAGGAGGAG GAGGAGGAGG AGGAGGAGGA

        70         80         90        100        110        120
GGAGGAGGGC TGCTGCTTCC AGGCATCTCC TCACCAACTG ATC....... ..........
```

Nucleotide sequence of clone pGMSSB12. See pages 187 and 296 for additional data.

```
10         20         30         40         50         60
CCACCACTGC TGCTTCTGCT GCTGCTGCTG CTGCCGCTGC TGCCACTCGT CACCGTGACG

        70         80         90        100        110        120
CCGCTGACGC TGCTGCTGCT GCTGCTGCTG CTGCTGCTGC TGCTGCTGCT GCTGCTGCTG

       130        140        150        160        170        180
CTGCTCTCCC TCGCCCCGCC TGCCCGGGCC GCCCGCACTT CG........ ..........
```

Nucleotide sequence of clone pGMSSB19. See pages 187 and 296 for additional data.

```
10         20         30         40         50         60
GCGCCTCTCC TTGCCTCAAC GGCCTTTGGC TCCTCCTCCT CCTCCTCCTG CTGCTGCTGC

         70         80         90        100        110        120
TGCTGCTGCT GCTGCTGCTG CTGCTGCTGC TGCTCTCCCT CGCCCCGCCT GCCCGGGCCG

        130        140        150        160        170        180
CCCGCACTTC GCG....... .......... .......... .......... ..........
```

Nucleotide sequence of clone pGMS14H. See pages 192 and 296 for additional data. Note the *Hin*PI1 (GCGC) restriction endonuclease site at nucleotide 150.

```
10          20          30          40          50          60
GCGCGAAGCC GCCGCAGCCC GCCACCGGCC GCCGGGAAAG TTAGTGCGAG AGGGGCCGGG

         70          80          90         100         110         120
AGGAAGAGGA GGAGGAGGAG GAGGAGGAGG AGAGAGGAGG AGCAGCAGGA GCAGCAGCAG

        130         140         150         160         170         180
CAGCAGCAGC AGCAGCAGCA GCAGCAGCAG CGCCTCCCAT TGCCTCCTAG TGCCTCCTCC

        190         200         210         220         230         240
TCCTCCTCAC GCCTCTTCTG CCTCCTCG.. .......... .......... ..........
```

Nucleotide sequence of clone pGMS23H. See pages 192 and 296 for additional data. Note: beginning at nucleotide 185 this sequence is homologous to the domestic chicken (*Gallus gallus*) voltage-gated calcium channel (see page 192).

```
10         20         30         40         50         60
CGAAGGTGCG GTAGGAGGCA AGAAGAGGAG GAGGAGGAGG AGGCGAGAGA GAGCAAGAAG

         70          80          90         100        110         120
GCGGCACGAG CAGCAGCAGC AGCAGCAGCA GCAGCAGCAG CAGCAGGAGG AGGAGGAGGA

       130         140         150         160        170         180
GGAGGAGGAG GAGGGGGACT GAGGAGCAGG CGGCGGCAGC AGCAGCAGCC GGGTTGCTAG

       190         200         210         220        230         240
GGATGGAGAG ATCCCGTGTC ACCCGGATGT GAGTGTCATG TTGGCCAAAC TCTGAGGGAT

       250         260         270         280        290         300
TTGGCAGCGG CGAACGATCA GCAGCAGCAG GAGGAGGAGG AGGAGGAGGA GCAGCCCCTC

       310         320         330         340        350         360
G......... .......... .......... .......... .......... ..........
```

Nucleotide sequence of clone pGMS3/31. See pages 206 and 296 for additional data.

```
10         20         30         40         50         60
GATATACAGC AGGCCCAAAT AGTTCACAGT CAAGGCATCT GATACAGAAA ACACTTGTGT

        70         80         90        100        110        120
TTCTGAATAA CATTATGTTC TCACATCATC ATCATCATCA TCATCATCAA TGATGATGAT

       130        140        150        160        170        180
GATGATGATG ATGATGATGT CGGTGGGCAC AGTGACTGCG TGATGGATGA TGGTGATGAA

       190        200        210        220        230        240
GATGAAGATT ATGAAGATGA TGATGATGAT GATGATGATG ATGATGGTGA TGTTGGTGGG

       250        260        270        280        290        300
CACAGTGACT GTGTGATGGA TGATGGTGAT GATGATGGTG ATGGTGGGCT CAGTGAACTA

       310        320        330        340        350        360
CGTGATGAAT GCCCAAGAGC ATGGGGAGGG CCAGGGCAGA GCACCAGCGG CAGTGGGTGA

       370        380        390        400        410        420
CACAGGGTGG GCTTTGGAGC GAGGAGGGAC AAACAGCAGG CACTGTAGCC TGCATCACAG

       430        440        450        460        470        480
AAACAGGGTC CTGTTTGTGC CCCTCAGTCC TGCACAGACC AAGGAGATGC ACTACATGCT
```

Nucleotide sequence of clone pGMS3/46. See pages 207 and 296 for additional data.

```
10         20         30         40         50         60
GATCTGCAGC CCAGTGTTGG CTTACTGGAG GAGAACCAGC TCATGGCAAG ATGATGATGA

         70         80         90        100        110        120
TGATGATGAT GATGATGATG ATGATGATGA TGATGGAGAT GAGGGGATTT ATTGTGAACC

        130        140        150        160        170        180
CTTCCCTCAG CACCATGCAA GGGGGCAGTT ACCCAGAGCA GCCGAACAGC TGTGACTCTG

        190        200        210        220        230        240
CCTACTTGGC TGATC..... .......... .......... .......... ..........
```

Nucleotide sequence of clone pGMS3/47. See pages 207 and 296 for additional data.

```
10         20         30         40         50         60
GATCAGCCAA GTAGGCAGAG TCACAGCTGT TCGGCTGCTC TGGGTAACTG CCCCCTTGCA

        70         80         90        100        110        120
TGGTGCTGAG GGAAGGGTTC ACAATAAATC CCCCTCATCT CCATCATCAT CATCATCATC

       130        140        150        160        170        180
ATCATCATCA TCATCATCAT CATCATCATC ATCATCATCA TCATCCGCCT CTGCTCATCA

       190        200        210        220        230        240
GGAAAGGTTG TAGCTGGAAA ACAAGAGATC .......... .......... ..........
```

Nucleotide sequence of clone pGMS3/40. See pages 209 and 296 for additional data.

```
10          20          30          40          50          60
GGTATCGCGT GTGCAAGGCC CAGGGTGACC CCCGTGCACG GTGTGGGTCA GCCCGTGTGT

         70          80          90         100         110         120
GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTAAAGCA CGGGGTGGCC CCTGCG....
```

Nucleotide sequence of clone pGMS16/38. See pages 211 and 296 for additional data.

```
10         20         30         40         50         60
GATCAGAAGT GATTCAGGCC CCCAGGCAAA GAAACAAACA AATGCAGGAC TACGTGTGTG

        70         80         90        100        110        120
TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTGTG

       130        140        150        160        170        180
TGTGTGTGTA TGCGTCTGTG TGTGGTTCTA AATGAACTGC TTATGCAGGA GATACCCCGA

       190        200        210        220        230        240
TGATC..... .......... .......... .......... .......... ..........
```

Nucleotide sequence of clone pGMS16/4B. See pages 212 and 296 for additional data.

```
10         20         30         40         50         60
GATCCCGCTG GCTGGAAATT GTTGGCCAGA GCAGAGGCTG CAGGTTGCGG GCTGTTGTTT

        70         80         90        100        110        120
GCCTCAGGGG TGGTGGTGGA GGTGGTGGCG GTGGCGGCTG CTGCTGCTGC TGCTGCTGCT

       130        140        150        160        170        180
GCTGCTGAGC ACTCCTCTGT GCCCGTTTCT GGGAAAGCAG CCAGTCTAGT CTTGAGAGCC

       190        200        210        220        230        240
TTCTTGAGCA ACCACCACAT GACCTGCGAT TAACCACCTC CTCCAGTCTG CCCTGGCCTG

       250        260        270        280        290        300
CCGCTTCAAG CACTGCCTTT TCAGCTCGTG TGCTGCAGAA ATAGGTGGGC CCTGTCTGCA

       310        320        330        340        350        360
GCTGTGGGCG AGAGCACCTC CAGCGACAGC TGGTTCCTGG AGGGGTCTGC AGAGCTGGTG

       370        380        390        400        410        420
ACTCGTGTCT GCAAGGATGA AGCAATGTGC TGGCGGCCCG TGAAAGGATA CTGCAACGCT

       430        440        450        460        470        480
ATCTGCTGCA GCTCTCTGCC AGGAGCCCGC CGGATC.... .......... ..........
```

Nucleotide sequence of clone pGMS16/12B. See pages 214 and 296 for additional data.

```
10         20         30         40         50         60
GATCTGTCAT GGATTAACCC CAGCCGGCAA CTAAGCACCA TAGAGTCGCT TGCTCACTCC

         70         80         90        100        110        120
ATGCCCCAGT GGGACGGGGG AGAGAATCAG AAGGGTAAAA GGGAGAAAAC TCATGGGTTG

        130        140        150        160        170        180
AGATAAGAAC AGTTTAATAA TTAAAGTAAG TAAATAGAGT AGTAGTAATA GTAGTAGTAA

        190        200        210        220        230        240
TGATAATAAT GATAATAATA TAAATGATAA TGATAATGAT AATGATGATG ATGGTGATGA

        250        260        270        280        290        300
TGATGATGAT GATGATGATG ATGATGATGA TGATGGGACT CAGAGACTGA CAGACCTGTC

        310        320        330        340        350        360
TTAGAGCAGC TCCCTTATAA CTCTCATCTG CACGATC... .......... ..........
```

Nucleotide sequence of clone pGMS16/29B. See pages 214 and 296 for additional data.

```
10         20         30         40         50         60
GCCGGTAGTT GAGGGCACGT GGCACTTTGC AAGAGATGTT TAAGCATTTA ATAGGCTCTT

        70         80         90        100        110        120
GCCCAGACTG AGCCGCAGCA GACAGTGGCA GCATCAAGAA TAAAACAGTT CCTGAATGCT

       130        140        150        160        170        180
GAACCCATTA TTCCCCATGA ACCCCACCCC AAGCTATCTC ATATCGATGA CTCGCCTTTG

       190        200        210        220        230        240
CAGCAGCAGC AGCAGCAGCA GCAGCAGCAC CTGCCTTTCC CAGGCTTCCC ATGCGTGCTC

       250        260        270        280        290        300
TTAATCAGTT CTGCCTCACC CCCCCC.... .......... .......... ..........
```

Nucleotide sequence of clone pGMS16/45B. See pages 218 and 296 for additional data.

```
10         20         30         40         50         60
CGGTAGTCGC ATGGAGGGGG GCAGCTTGTC CATGGCCGAT GCTGCTGCTG CTGCTGCTGC

        70         80         90        100        110        120
TGCTGCTGCT GCTGCTGCTG CTCAGTTAGA GGGAGAGACC CCCGTTCTGC CCAGGAGGGG

       130        140        150        160        170        180
ACCGTCACTG CCCCCAGGCC CCTGCCCCCA GCTGCAAGGG GCAGCAGGGA CCAGAGACTG

       190        200        210        220        230        240
GCGTGTCTTC TCCTTACCAG ACTGTGGCAT CCACGGGCTC TGCATCGAAA CAGAGGCTCT

       250        260        270        280        290        300
GCATCAGAAG GGCAGGAAAA AAAAGAGGGA AGGCAAAAAA GC........ ..........
```

Nucleotide sequence of clone pGMSZapEco[B]1/1. See pages 233 and 296 for additional data.

```
10         20         30         40         50         60
AATTGCACTG TGCATCACTT ATTCGGTATA TTCTTTTATC ATCATCATCA TCATCATCAT

        70         80         90        100        110        120
CATCATCATC CTTATTATCC CCCTTCCTTT TTTCTGTCCT ATTAAACTGT CTTTATGTTA

       130        140        150        160        170        180
ACCCACAAAT CTTACTTCTG CTGCCCCCCC CACCCCCCAC TCTTTCCCCC ATCCCACTGC

       190        200        210        220        230        240
AAGGGGGAGT GAGCAAATGG CTGTGTGGTT TTTAGCTGCC TGCTGGCTTA AACCATGACA

       250        260        270        280        290        300
CAGTGTTTCA TGTCTAATGG TTTGGAATAT GAAGAAAACA TGAAATAATT ..........
```

Nucleotide sequence of clone pGMSE[2]1/1. See pages 235 and 296 for additional data.

```
10          20          30          40          50          60
AATTACTCAG GGAGAGGAGA GGAGAGGAGA GGAGAGGAGA GGAGAGGAGA GGAGAGGCGA

         70          80          90         100         110         120
GGAGAGGAGA GGAGAGGAGA GGAGAGGAGA GGAGAGGAGA GGAGAGGAGA GGAGAGGAGA

        130         140         150         160         170         180
GGAGAGGAGG AGACCTTTCC ATCCAGGTTC CCTTGGGTCA GCAGCAGATG CCTCAGCACA

        190         200         210         220         230         240
GTCTATTTTC TGCTTTCAGC AAAACAGGTG CAATCTTTGG GCAATATTTT GAGCTC....
```

Nucleotide sequence of clone pGMSE[2]2/1. See pages 235 and 296 for additional data.

```
10         20         30         40         50         60
AATTTTCCCT GGGCTCCTCC TCCAACAGCA TCCAGGACAA AGGAAGAAAA GGGGAAGTGC

         70          80          90         100        110        120
CTGAGGAAAC AACTGACATG CAAAATGTCT AGGGAAACAG AGGGAAAGCA AGAGATAAGC

        130        140        150        160        170        180
AGGTTCAGAA AATGTTCAAA TGGCTTAAGT TTTTCTCGTC TCTATTGCCT GGAGAAGTGC

        190        200        210        220        230        240
TTTTCATCTA TGCATGTAAA AGCACTGAAA CTCTTCTAAA TAGCTGTGCT CCCTCTTTCT

        250        260        270        280        290        300
CCTCCTCCTC CTCCTCCTCC CAGGTAGGAA GAGAAAATAA AATT...... ..........
```

Nucleotide sequence of clone pGMS[B]4. See pages 239 and 296 for additional data.

```
10         20         30         40         50         60
TTTTCTAAAG TCAGTGCTCC AAGGTAAGTA TAACAGCAAA CCTGACAAGA GCATTTTGCC

        70         80         90        100        110        120
TTAATAAGGC AGTATTTTTA AAATATTTTG TTTTCCAGTT CTAGTAGGAT CTGTGATAAC

       130        140        150        160        170        180
ACATGCAACT TGGAACTTAA TTGGTCCGAG AGAACAGACA TCATCATCAT CATCATCATC

       190        200        210        220        230        240
ATCTATTGGC TGTCCCAGGT GAAACCCAGG TATTAACAGT GTCTATTGGC TGCTCGAAGT

       250        260        270        280        290        300
GCTGGTGTCC TACAGCCTCT TAGGTCTCTA TGATACAAAC CCGACTTTCT ATCCAAAACG

       310        320        330        340        350        360
GGGCTTCTCT TCCCGT.... .......... .......... .......... ..........
```

Nucleotide sequence of clone pGMS[2]3. See pages 239 and 296 for additional data.

```
10         20         30         40         50         60
CCAGGACAGC TGACCCAAAC TGGCCAAAGG GCTATTCCAT ACCATACGAC GTCATGCTCA

        70         80         90        100        110        120
GTATATAAAC GGGGGGAGTT GGCCGGGGGG AGGAACCGCT GCTCAGGAAC TGGCTGGGCA

       130        140        150        160        170        180
TCGTTTGGCA TGTGGTGAGC GATTGCATTG TGCATCTCTT GTTTTGTATA TTCTTTCATC

       190        200        210        220        230        240
ATCATCATCA TCATCATCAT TATTATTTCC CTTCATTTTC TGTCCTATTA AACTGTCTTT

       250        260        270        280        290        300
ATCTCGACCC GTGAATT... .......... .......... .......... ..........
```

Nucleotide sequence of clone pGMS3Δ5. See pages 242 and 296 for additional data.

```
10         20         30         40         50         60
CCCAGCAGCA GGTGAAGCTG AATCAACAGA GTCATCCTTT TGTATTTCAG TAAACTCAGC

         70         80         90        100        110        120
CAAGGACTGA TGGTAATCTG TAGGCTATTT CTTCCTCTCC TCTCCTCTCC TCTCCTCTCC

        130        140        150        160        170        180
TCTCCTCTCC TTCTCTTCTC TCTTTTCTTT TCTCCTCTCC CTCTCCCTNN NNNNAGGGGC

        190        200        210        220        230        240
AGGGGAGAGG AGAGGAGAGG AGAGGAGAGG AGAGGAGGGA GAGGAGAGGA GAGGAGAGGG

        250        260        270        280        290        300
GAGGAATGGA ATTGATAGCA GCAATAAAAA CTGTTTGGCA GCTCCCTGGT CAGCACAAAT

        310        320        330        340        350        360
ACTTGTAGCA AGTAATTCTA G......... .......... .......... ..........
```

Nucleotide sequence of clone pGMSE[4]1/2. See pages 243 and 296 for additional data.

```
10         20         30         40         50         60
AATTACTTTT CACCCTTTGG TCTCAGTTTT CCTGCACCCA TACTCAGATT TGGTAAGTCT

         70         80         90        100        110        120
GACACCACTA CCTAGGCAGT CTTGGCCTTA TGTGGTTTTA CTGACAGGGT TAAACTGTTC

        130        140        150        160        170        180
TCTAGTGGTT CTGCAAGGCT CTGTGCCCCA AAAAGGGNNN NNNAAACGCT GTCCTTCCTT

        190        200        210        220        230        240
CCCTCCCAGA CCCACTGCCC TCGGTTAGCA TCGCACCAAA CCCACCTTAT TCTCCCTTTC

        250        260        270        280        290        300
TCCTTGTTGC GTTGTCCTAC TCGGTTAGTT GATTTGATGT AAAGATGACG GTGTAATT..
```

Nucleotide sequence of clone pGMSZapEco[B]3/1. See pages 243 and 296 for additional data.

```
10         20         30         40         50         60
TTTCCAGAAC TGTCACCATT GAAATATTCC TTTTGCTTAA AGCTACATTA TGAAACAGTA

        70         80         90        100        110        120
ATTAAAAAAG ATGCAAATAT ACCTTTACAT ATGCATCAGT ATTAAAAATA ATACACGCTG

       130        140        150        160        170        180
GAGCCAACAG GAGCCCAGGA GGAGGAGGAN AGGANAGGAN NNNNGAGGAG AATGGCGCGG

       190        200        210        220        230        240
CGGTGGAGCC CGACCGGAGC CGCCCTCTCC CCGCCGGGCT CTTCTCGGCC CGGATTGCTC

       250        260        270        280        290        300
GTTTCGGGGA TGTTTGTTTT GTTGTT.... .......... .......... ..........
```

Partial nucleotide sequence of clone pGMSBΔB. This sequence originates from the T3 primer. The distal nucleotide sequence was determined by lslabs.com.

```
10         20         30         40         50         60
GATCTGGTGC ACCAGTATGG CACCGGGAGG CTTTCAAGGG TGCCGGTAAG GCACTGGGAG

         70          80          90         100        110        120
CAGCTCTGGG GCACCAGTAA GGCACTGGGA GCAGCTCTGG GGCACCAGTA AGGTACTGGG

        130        140        150        160        170        180
AGCAGCTCTG GGGCACCAGG AAGGGACTGG GAGCAGCTCT GGGGCACCAG TAAGGCACTG

        190        200        210        220        230        240
GGAGCAGCTC TGGGGCACCA GTAAGGCACT GGGAGGCTTT CAAGTATGCC AGTAAGGGAC

        250        260        270        280        290        300
TGGGAGCAGC TCTGGGGCAC CAGTAAGGCA CTGGGAGCAG CTCTGGGTCA CCAGTGTGGC

        310        320        330        340        350        360
ACCAGGACGC TTTCAAGGGT GCCAGCAGGG GACTGGGAGC ANCTCTGAGG CACCAGTAAG

        370        380        390        400        410        420
GGACTGGGAG CAGCTCTGGG GCACCAATAA GGCACTGGGA NCANCTCTGG GGCACCAATA

        430        440        450        460        470        480
ANGCACTGGG AGGCTTTCAA GGGTGCCCNT AANGCACTGG GAACANCTCT GGGGCCACCA

        490        500        510        520        530        540
ANNAAGCACT NGGNANCANC TCTGCNGCAC CANTAANGCA CTTGGGAACA NCTTNTGGGG

        550        560        570        580        590        600
CACCANNANG GCNACNGGGG AANGCTTTTN AAGGGTNCCC CCNNAAAGGG AACTNGGGAA

        610        620        630        640        650        660
ACNNNCTCTN GGGGCNACCC ANNNAAAGNC ANNTNNNNAA CNACNTCTTN GGGNCNNCCA

        670        680        690        700        710        720
NNNAATTNNN NACNTANNNA ANCCCTTTNN AA........ .......... ..........
```

Nucleotide sequence of clone pGMS04/6F. The STR ended at the MCS.

```
10         20         30         40         50         60
ATCCTTCCTC CAAATCACCT TAACTCTGCC CATCAGAGCA GATGCAGAAC CACACAGGAG

        70         80         90        100        110        120
TGGGGAGGGC TAGACCCAGA AGCAGGCACA CAGGAGACCA AAGAAGAAGA TGATGATGAT

       130        140        150        160        170        180
GATGATGATG .......... .......... .......... .......... ..........
```

Nucleotide sequence of clone pGMS3/21R. The STR ended at the MCS.

```
10         20         30         40         50         60
ACGCCCACCC ACTGTGGGCT CTCCTCGCCC CACACACTCC TCACACACCC TGTGGCATCG

        70         80         90        100        110        120
GTCACCAAGC GTCATAGCCA TCACCACCAC CATCATCATC ATCATATCAT CATCATCATC

       130        140        150        160        170        180
ATCATCATCA TCATCATCAT CATCATCATC ATCATCATCA TCATCATCAT CATCATCATC

       190        200        210        220        230        240
ATCATCATCA TCATCATCAT CATCATCATC ATCATCATCA TCATCATCAT CATCATCATC

       250        260        270        280        290        300
ATCATCATCA TCATCATCAT CATCATCATC ATCATCATCA TCATCATCAT CATCATCATC

       310        320        330        340        350        360
ATCATCATCA TCATCATCAT CATCATCATC ATCATCATCA TCATCATCAT CATCATCATC

       370        380        390        400        410        420
ATCATCATCA TCATCATCAT CATCATCATC ATCATCATCA TCATCATCAT CATCATCATC

       430        440        450        460        470        480
ATCATCATCA TCATCAT... .......... .......... .......... ..........
```

Nucleotide sequence of clone pGMS45WF.

```
10         20         30         40         50         60
GATCTTTGGT TGGCAGGTGG TGAGCAATTG CATTGTGCAT CACTTATTTT GTATATTCTT

        70         80         90        100        110        120
TTATCATCAT CATCATCATC ATCATCATCA TCATCATCAT CATCATCATC ATCATCATCA

       130        140        150        160        170        180
TGTCATCATC ATCATCATCA TCATCATCAT CATCATCATC TCATCATCAT CATCATCATC

       190        200        210        220        230        240
ATCATCATGA TGATGATGAT GATGATGATG ATGATGATGA CGATGATGAT GATGATGATG

       250        260        270        280        290        300
ATGATGATGA TGATGATGTT GTTTGGAAGG AGAGGGGAGG CCCGATTTTC TGTTCCGTTA

       310        320        330        340        350        360
ATTGGGATC. .......... .......... .......... .......... ..........
```

Nucleotide sequence of clone pGMS16/8R.

```
10         20         30         40         50         60
GATCCGAGCG TGACCCTCTG CAAATTTACG CAGTGATTTT GCTGCTGCTG CTGCTGCTGC

         70         80         90        100        110        120
TGCTGAGGGG TGGGAGTGGT CCTGCCTCGT GTAGGATGAT AGTCTTGAAA CCTTAGATTA

        130        140        150        160        170        180
AGCACGCGCA GTCAATCTCC TCGGGACTTC CCGGTGTGCG TTCGCACGCA CGTATGTATG

        190        200        210        220        230        240
TAAACAGCGA ACGTCTGAGC AGTGACCGTA ATGAGTTAAT AGAAAGTACT AACATCGCGT

        250        260        270        280        290        300
AAGAGAAATC AGCGTTTCGG GCTTTGTGTT TGGGTATCTG GTGATC.... ..........
```

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