PCR-based Method for Sexing Roseate Terns (*Sterna dougallii*)

Thomas J. Sabo, [1] Rick Kesseli, [1] Joy L. Halverson, [2]
Ian C. T. Nisbet, [3] and Jeremy J. Hatch[1]
[1]*Department of Biology, University of Massachusetts, Boston, Massachusetts 02125, USA;*
[2]*Zoogen Inc., 1105 Kennedy Place, Suite 4, Davis, California 95616, USA; and*
[3]*150 Alder Lane, North Falmouth, Massachusetts 02556, USA*

A simple method for identifying the sex of sexually monomorphic birds would be widely applicable to studies of population-genetic structure, demography, and behavior. In this paper we describe a method for identifying W-chromosome-specific genetic markers and show that these markers can be used to identify sex from very small samples of tissue, such as single mature feathers and, thus, from individuals of any age. We have applied this technique to Roseate Terns (*Sterna dougallii*) and further shown that the marker correctly identified sex in a closely related species, the Common Tern (*Sterna hirundo*). This method can be used to develop markers for other groups of birds, or any organism with chromosomal sex determination.

The Roseate Tern is a cosmopolitan species of seabird that occurs in widely separated breeding populations in temperate and tropical regions of the Atlantic, Indian, and West Pacific oceans (Cramp 1985). The population in the northwestern Atlantic, which is the subject of our study, is listed as endangered (U.S. Fish and Wildlife Service 1987). Preliminary observations indicate a female-biased sex ratio in this population (Hatch and Nisbet pers. obs.). Information about the sex of many more individuals at various life cycle stages will be important for characterizing sex-specific behavior and investigating this female-biased population sex ratio.

Like many terns and other seabirds, the Roseate Tern is sexually monomorphic in plumage, and the size differences between males and females are small and not well characterized. Sex-specific behaviors (copulation, courtship feeding) in this species are usually performed away from the nest site and only occasionally can be used to identify the sex of individual birds. Alternative methods for sexing include laparoscopy and handling the adults during the period of egg laying when morphological differences are distinct. However, both of these methods pose unacceptable risks of injury or nest abandonment for members of this endangered population. Another method for sexing birds based on analysis of tissue samples is karyotyping from fibroblasts grown from blood-feather pulp (Parker et al. 1991). This method is laborious and has not been used on terns.

Molecular techniques for sexing used on several bird species have been based on hybridizing labeled probes to genomic DNA. The probes are either W-chromosome-specific or they identify multigene families with W-specific members (Griffiths and Holland 1990, Quinn et al. 1990, Dvorak et al. 1992, Griffiths 1992, Millar et al. 1992, Graves et al. 1993, Longmire et al. 1993). These molecular methods are slow and laborious, they require relatively large quantities of DNA, and most of them involve use of radioisotopes. Such methods are neither economical nor practical for field studies involving large sample sizes or nestlings. With the advent of random-amplified polymorphic DNA (RAPD; Williams et al. 1990), a new source of easily accessible genetic markers is available. These markers have been applied to genetic studies in plants (Tingey et al. 1992) and, recently, have been used for sexing birds (Griffiths and Tiwari 1993).

We describe a method for sexing Roseate Terns based on the polymerase chain reaction (PCR) that offers considerable advantages in technical simplicity and time. The method was developed with blood samples from adult Roseate Terns. However, because only nanogram quantities of DNA are required, the method is much more versatile and works with single mature feathers.

Genetic markers specific to the W chromosome are identified by separately pooling samples of male and female DNA, and then screening the two pools for RAPDs (Michelmore et al. 1991). A typical RAPD reaction yields 6 to 10 bands (loci) that are produced by amplification with one arbitrary 10-base primer. Once a W-linked RAPD marker has been identified, it can be converted to a more specific and reliable PCR-based marker (i.e. a sequence-characterized amplified region, SCAR; Paran and Michelmore 1992) by increasing the length of the original primer at each end of the targeted region. The high specificity of a SCAR marker overcomes several of the drawbacks of RAPDs (Kesseli et al. 1992, 1994, Paran and Michelmore 1992).

Methods.—Roseate Terns initially were sexed using an RFLP probe (Zoogen Inc., Davis, California). The method was verified using field-sexed Common Terns. The Roseate Terns sexed by this method were used to identify RAPD markers and develop SCAR primers. Finally, these primers were tested on a larger sample of field-sexed Common Terns.

Terns were trapped at nests on Bird Island, Marion, Massachusetts (41°40′N, 70°43′W). Roseate Terns were trapped shortly before their eggs hatched. Common Terns were trapped within 24 h after the first egg was

laid; at this time, females were gravid with subsequent eggs in the clutch, and adults were sexed by the presence or absence of a palpable egg in the oviduct, or by body mass. In our studies, we have found that Common Terns over 140 g at this stage in the laying cycle were females, and birds under 130 g were males. DNA was extracted from samples of blood following the protocol of Dvorak et al. (1992), and from 2 to 5 mm of the calamus (shaft), including its tip, of single breast feathers following the protocol of Leeton et al. (1993).

The ^{32}P labeled probe *DZWM1* (Zoogen Inc., Davis, California) was hybridized to Southern blots of samples from each species cut with *Sac*1. This RFLP probe identifies a single gene which is both Z and W chromosome-linked in a wide variety of bird species and in most species displays sex dimorphism (Dvorak et al. 1992).

To identify RAPD markers specific to the W chromosome, we employed the technique of bulked segregant analysis (Michelmore et al. 1991, Mulcahy et al. 1992). Separate pools of DNA from 12 male and 12 female Roseate Terns were constructed and screened for RAPDs following the protocol of Williams et al. (1990). Amplified samples were electrophoresed on 2% agarose gels in 1× TBE buffer for 3.5 h at 70 mA and then stained with ethidium bromide. Ten-base primers (Operon Technologies, Alameda, California) that produced bands in the female pool and not in the male pool (Fig. 1A) were tested on the 24 individual DNA samples. If the band was found in all females and no males, it was considered W-linked.

To develop the SCAR, the band was excised from the gel and the DNA was purified using Gene Clean II (Bio 101 Inc., La Jolla, California). The DNA was reamplified using the same primer and purified again. The fragment was cloned using the pT7Blue T-Vector from Novagen (Madison, Wisconsin). To verify that the correct sequence had been cloned, the amplified fragment was used as a labeled probe and hybridized to a Southern blot of the RAPD profile. The probe hybridized to bands of correct size in the females only. The SCAR primers were developed by sequencing the two ends of the cloned RAPD fragment. Two new primers, forward and reverse, were produced (Operon Technologies). They contain the first 10 bases of the original primer used to detect the RAPD, along with an additional 19 or 20 bases. This increased length, along with a high annealing temperature, ensures the amplification of only the specific W chromosome marker, not the 6 to 10 bands often found with RAPDs.

Samples of known and unknown sex were screened for presence of the SCAR specific to the W chromosome. The reaction mixtures were in volumes of 25 μl containing 2.5 μl of 10× buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl$_2$, 0.1% gelatin), 2.0 μl dNTP (1.25 mM each of dATP, dCTP, dGTP and dTTP), 0.25 μl of the forward and reverse primers (2.5

μM), 12.5 ng of DNA and 1 unit *Taq* polymerase. The PCR conditions were as follows: initial DNA denaturation at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min; primer annealing at 65°C for 1 min and primer extension at 72°C for 1 min. To produce positive controls we used reduced annealing temperatures (55°C or less) to generate faint, nonspecific bands in males (see Results). Amplification products were analyzed by electrophoresis under the conditions stated above.

Results.—The Zoogen probe *DZWM1* correctly identified the sex of 21 Common Terns of known sex. Two additional specimens that gave contradictory results were from birds that were handled at the same time, and it is likely that the samples were mislabeled in the field. These two samples are excluded from subsequent analysis. The 52 Roseate Terns evaluated produced banding patterns identical to these Common Terns and were sexed on this basis. Two separate pools of Roseate Tern DNA were constructed from 12 females and 12 males selected for similar DNA quantity and quality as determined from hybridization to the Southern Blot.

From 180 10-base primers (Operon Technologies), we identified approximately 1,400 readable bands, 6 of which distinguished the pools. Of these six bands, two (OPj18, 1,720 bp and OPo16, 615 bp) were W-linked; they were present in all 12 female individuals and in none of the 12 males (Fig. 1A). The other four bands were polymorphic and generally more frequent in females than males, but not W-linked. Fifty-two samples scored for sex in the RFLP study and retested with the RAPD primers OPj18 and OPo16 were all correctly identified.

We were unable to clone band 1720 from primer OPj18; large fragments are generally more difficult to clone and specific sequences within fragments may inhibit the cloning. The RAPD locus *OPo16*[615] was successfully converted to SCAR (*SCo16*[615]). The extended primers for this SCAR are as follows: Forward (SCo16F), 5'-TCGGCGGTTCATAAACCCATCAG-TTGGGG-3' (29 bases); Reverse (SCo16R), 5'-TCG-GCGGTTCCGTAATCGTGTGTGTATATT-3' (30 bases). We tested these SCAR primers on all 21 Roseate Terns sexed by Zoogen (with RFLP probe *DZWM1*) and correctly identified the sex of all individuals. In addition, the SCAR primers correctly identified sex in 45 Common Tern samples sexed by morphology (Fig. 1B).

The absence of a band indicates either maleness or a faulty reaction. Lowering the annealing temperature creates an internal control that distinguishes between these possibilities. At 55°C mismatching occurred and a larger (ca. 2,100 base pairs [bp]), faint band in the males was amplified while maintaining an intense W-specific marker in the females. Presence of this 2,100-bp band without the 615-bp band indicated that all components of the reaction were present and that the individual was male (Fig. 1B). Keeping
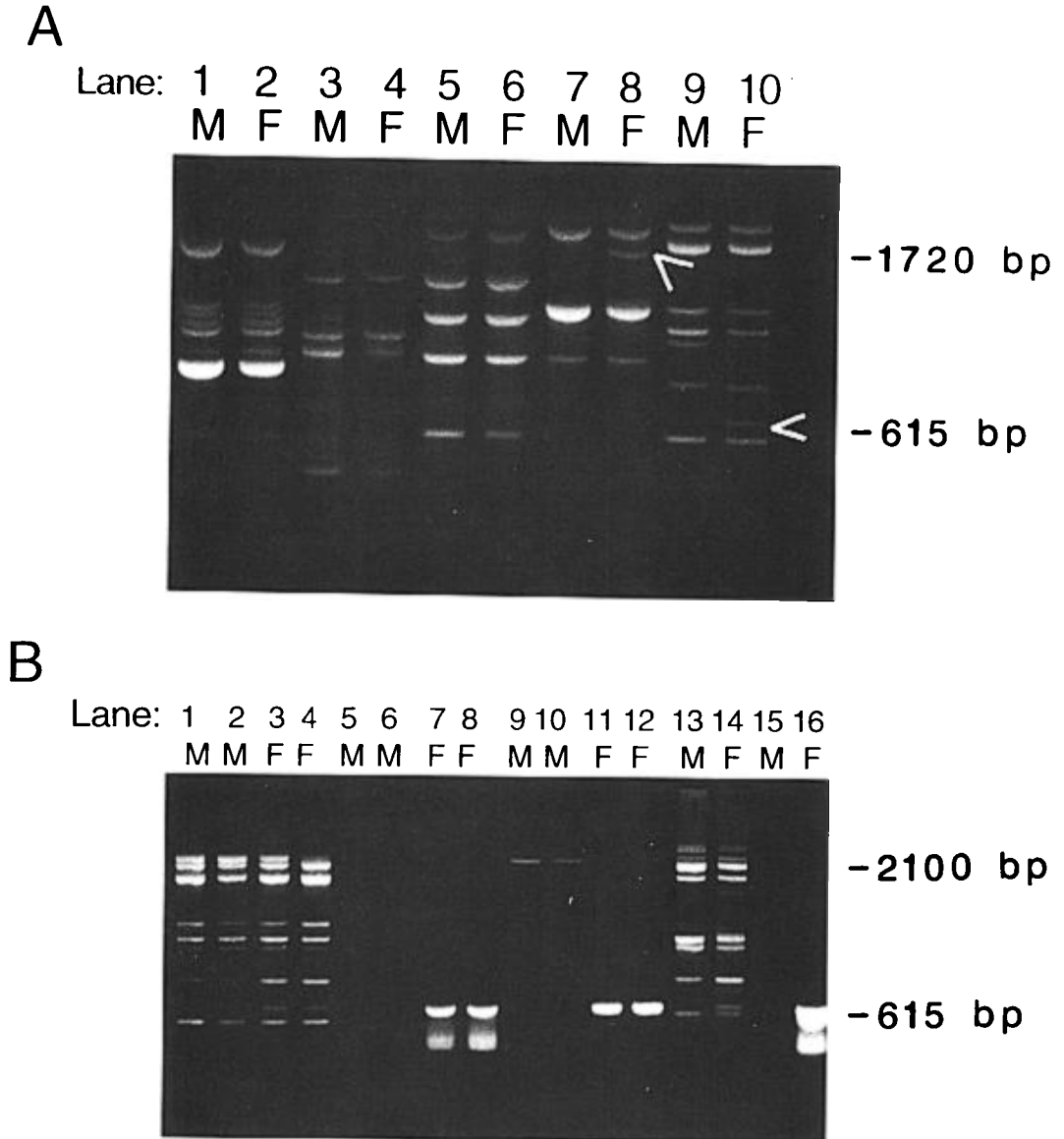
Fig. 1.   Agarose gels stained with ethidium bromide demonstrating (A) bulked segregant analysis and (B) RAPDs and SCARs. (A) Pools of DNA from 12 male [M] and 12 female [F] Roseate Terns screened for RAPDs. DNA of each pair of pools amplified by five different 10-base primers. Operon primers OPj18 (lanes 7 and 8) and OPo16 (lanes 9 and 10) produced bands 1,720 bp and 615 bp, respectively, that were present in female pool and not in male pool (arrows). These bands subsequently shown to be present in every female and absent in every male, hence *W*-linked. (B) Lanes 1 to 12 (6 males [M] and 6 females [F]) contain amplified DNA of Roseate Terns. Lanes 13 to 16 contain amplified DNA (2 M and 2 F) of Common Terns. Lanes 1 to 4, 13, and 14 show fragments amplified with primer OPo16. Band for RAPD *OPo16*[615] present only in females is identified. Lanes 5 to 8, 15, and 16 show fragments amplified with extended primers (SCo16F and SCo16R) at 65°C annealing temperature. Lanes 9 to 12 show same reactions for Roseate Terns at 55°C annealing temperature. A large band (2,100 bp) serves as internal control in males at 55°C. A smaller band is amplified with extended primers in some females (lanes 7, 8 and 16) and possibly represents a duplicated region on *W* chromosome.

the high annealing temperature at which no internal control is amplified could lead to incorrect scoring, so we recommend adjusting the annealing temperature until a positive control is achieved. If the annealing temperature is too low, however, the specificity of the SCAR will be lost and many bands will be amplified. An alternative strategy would be to develop primers for an autosomal or Z-specific locus of a different size. These primers would produce bands in both males and females and could be incorporated into a single reaction.

DNA extracted from single feathers (either growing or mature) yielded the 615 bp band when amplified with the SCAR primers. However, the internal control for males, developed with blood DNA, proved to be unreliable with feather DNA and a more specific marker is necessary.

Discussion.—We have identified a marker linked to the W chromosome that allows us to identify the sex of sexually monomorphic Roseate Terns. The approach used can be applied to any genetically based sex-determination system and, once this type of marker is developed, it can be particularly useful for field biologists.

The pooling procedure used to identify a female-specific marker was first used to identify markers linked to disease resistance genes in plants (Michelmore et al. 1991). The procedure, termed bulked segregant analysis (BSA), can be used to identify markers (e.g. RFLPs or RAPDs) linked to any gene that segregates in any population. A simplified modification of this procedure was used later to identify markers specific to the Y chromosome in dioecious plants (Mulcahy et al. 1992). Recently, Griffiths and Tiwari (1993) used the same procedure to identify W-chromosome-specific markers in three species of passerines.

RAPDs offer several advantages over RFLP assays (Tingey et al. 1992). They do not require DNA libraries. They utilize PCR so they require only nanogram quantities of DNA and do not require radioactivity. RAPDs are fast and technically simple, but have the serious drawback of being highly sensitive to reaction conditions (Kesseli et al. 1992, Ellsworth et al. 1993). Slight deviations in magnesium, primer, or DNA concentration can alter the banding pattern, possibly masking the marker. With greater specificity and higher annealing temperatures, SCARs offer a reproducible marker that is less sensitive to reaction conditions. This is advantageous for studying natural populations where the quality and quantity of DNA collected in the field often vary. SCARs are also faster than RAPDs, requiring fewer PCR cycles and one-half the gel-running time. SCARs require less DNA and, because of the higher specificity obtained with two extended primers, they are less affected by contaminants such as bacterial DNA. Such contamination may be important for tissues containing very little DNA, as is the case with mature feathers, which are

a desirable source of DNA for studies involving young or endangered birds.

Our SCAR primers amplify a 615-bp segment located on the W chromosome. With this marker, visualized by electrophoresis, we are now able to distinguish between male and female Roseate Terns. This will permit further investigation of the female-biased sex ratio among breeding adults at Bird Island, which is evident from the occurrence of supernormal clutches and unusual mating associations including female-female pairs (Hatch and Nisbet pers. obs.). Although supernormal clutches have been found in several species of gulls and terns, they usually occur at low frequencies (Conover 1984) and are not well understood. In general, there is little information available about the population sex ratios of bird species (Clutton-Brock 1986).

The SCAR primers, derived from Roseate Tern genomic sequences, were also used to identify sex in the Common Tern, and they may be applicable to other related species. This topic is being investigated. Because of the rapid evolution of sex chromosomes, it is likely that these primers will apply only to a small range of species. Similar SCAR primers can, however, be developed for other groups of birds.

The successful sexing of individuals with the low amounts of DNA from single mature feathers represents a significant advance. Sufficient DNA can readily be obtained from individuals of any age (chick to adult, although natal down may be an unsuitable source). Such samples could be collected in conjunction with banding programs, which could lead to greatly expanded knowledge of sex-specific patterns of movement and mortality.

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