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RAPD-PCR AMPLIFICATION OF DNA EXTRACTED FROM AVIAN BLOOD INFECTED WITH *HAEMOPROTEUS* FAILS TO PRODUCE FALSE POSITIVE MARKERS[1]

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Abstract. We tested whether contamination of avian blood by the parasite *Haemoproteus* could cause the amplification of false positive genetic markers generated by random amplified polymorphic DNA (RAPD). DNA was extracted from blood taken from five Rock Doves (*Columba livia*) infected with *Haemoproteus,* and one uninfected control individual. A drug treatment was administered to clear the infection, and a second DNA sample extracted. The parasitized and unparasitized DNA samples were used in RAPD reactions with 20 different 10-nucleotide primers, which produced 178 well-resolved markers. In no case were any of the reaction products specific to the parasitized samples, and in almost all cases the products

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were found consistently across all replications of infected and non-infected samples. The high ratio of avian to parasite DNA and the greater genome complexity of the host probably prevents the amplification of markers unique to parasite species such as *Haemoproteus.*

Key words: blood parasites, Columba livia, *DNA,* Haemoproteus, *random amplified polymorphic DNA, RAPD, Rock Dove.*

A variety of molecular genetic techniques have been employed in the study of avian population genetics and evolution. One technique for generating genetic markers directly from DNA is the application of the polymerase chain reaction (PCR) method using short, random-sequence oligonucleotides as primers, termed RAPD for random amplified polymorphic DNA (Williams et al. 1990). Several studies of avian population genetics have utilized RAPDs, and RAPD analysis has the potential to be especially useful to ornithologists because of the ease with which large amounts of DNA can be collected from blood samples. Birds, like other non-mammalian vertebrates, have nucleated erythrocytes, each with 2–3 pg of DNA (Petitte et al. 1994). Blood samples can be easily collected in the field and stored for some time without cryogenics (Seutin et al. 1991), and the DNA easily extracted and purified in the laboratory.

Standard PCR methods are known to be highly sensitive to contamination (Kwok and Higuchi 1989, Kitchin et al. 1990), and the anonymous nature of RAPD markers allows the possibility that markers could be generated from any type of DNA contaminant within the sample of avian DNA. We hypothesized that, for avian DNA extracted from samples of whole blood, a serious potential source of contamination will be hematozoan parasites. Over 35% of the birds in North America are believed to carry one or more species of avian hematozoa (Greiner et al. 1975). Because the lysis buffers, used to store and preserve blood (Seutin et al. 1991), or the extraction buffers will lyse any cell placed in it, any hematozoan present will presumably release its DNA in quantities proportional to the intensity of the infection.

Species of the blood parasite *Haemoproteus* are the most common of the wild bird parasites known as haemosporidians, which complete part of their life cycles within circulating blood cells (Garnham 1966). If the presence of a haemosporidian caused the amplification of RAPD markers specific to the parasitic genome, the use of avian blood samples as a source of DNA for population genetic studies would be rendered invalid as far as RAPD-PCR analysis is concerned. This paper presents the results of an experiment designed to test whether DNA extracted from samples of avian blood infected with *Haemoproteus* will cause the amplification of parasite specific RAPD markers.

METHODS

Blood samples were collected from nine Rock Doves (*Columba livia*) with part of each sample placed in a 1.5 ml microcentrifuge tube with 1.0 ml of a commercial cell lysis buffer (Gentra Systems Inc., Minneapolis, Minnesota), and the remainder retained in a heparinized hematocrit tube. The reserve was used to prepare three blood smear microscope slides, which were fixed with 100% methanol for 2 min, stained for 15 min in 0.02% (weight volume^{-1}) Giemsa stain, rinsed with water, and air dried overnight. The gametocyte stage of *Haemoproteus* occurs within the cell membranes of the erythrocytes and is easily detected in a prepared slide (Fallis and Bennett 1961). Intensities of *Haemoproteus* were determined by surveying a total of 2,000 erythrocytes from each individual, counting the number of infected cells, and calculating the mean number and standard deviation of infected cells per 100 erythrocytes (Godfrey et al. 1987). Blood samples were collected from each individual twice prior to the administration of a drug treatment. Five Rock Dove specimens testing positive for *Haemoproteus* (RD-1 through RD-5) and one testing negative (RD-6) were retained for the experiment. The mean (± SD) infection intensities within each individual ranged from 0.2 ± 0.1 to 6.7 ± 0.4 infected erythrocytes per 100 examined.

To clear the *Haemoproteus* infection, subjects were orally administered 25 mg kg^{-1} of chloroquine diphosphate and 1 mg kg^{-1} of primaquine diphosphate simultaneously at the initiation of the treatment (0 hr), followed by three doses of chloroquine diphosphate (15 mg kg^{-1}) at 12, 24, and 48 hr. Blood samples were taken from all individuals at the initiation of treatment (Day T), six days later (Day T+6) and nine days later (Day T+9). All individuals were found to be clear of *Haemoproteus* gametocytes at Day T+9 using the protocol described earlier. For samples collected on Day T+9, a more extensive survey was conducted to ensure that no *Haemoproteus* gametocytes were present; this involved scanning as many cells as possible in a 10-min period to search for infected cells. Only one post-treatment sample (RD-3) revealed any sign of infection—a single infected cell. This level of infection probably is inconsequential, but we regarded results from individual RD-3 to be uninformative.

DNA was extracted, using a commercial kit (Gentra Systems Inc.), from all samples taken on Day T+9, and from a single blood sample representing the infected state of each positive specimen, selected based on the highest level of *Haemoproteus* intensity prior to drug treatment. DNA also was extracted from samples taken from the uninfected bird (RD-6) on Day T and on Day T+9. Each DNA sample was washed with 70% ethanol, air-dried overnight, and resuspended in Tris-EDTA buffer. Concentrations were determined by spectrofluorometer and diluted to 20 ng μl^{-1}.

We initially surveyed 80 primers (Operon Technologies Inc., Alameda, California: OP-A, OP-B, OP-C, and OP-D series) for their usefulness in a population genetics investigation. Each primer consists of 10 nucleotides, 60 to 70% of which carry the bases G or C. Reactions were performed using three of the infected blood samples with each primer; each 25-μl reaction consisted of 10 mM Tris-HCl pH 8.3, 50 mM KCl, and 2.0 mM $MgCl_2$, 0.1 mM each of dATP, dTTP, dCTP, and dGTP (Pharmacia Biotech, Piscataway, New Jersey); 0.2 μM of primer, 0.5 U of Taq DNA polymerase (Boehringer Mannheim, Indianapolis, Indiana), and 20 ng of DNA sample. Reactions were

TABLE 1. The 20 primers selected for the RAPD-PCR reactions. "Total number of scorable bands" is the number of bands determined to be easily and reliably scored in a population-level analysis. "Number of monomorphic bands" is the number amplified and scored in all six individuals across all replications.

Primer	Nucleotide sequences	Total number of scorable bands	Number of monomorphic bands	Number of polymorphic bands
OPA-2	5′-TGCCGAGCTG-3′	7	6	1
OPA-5	5′-AGGGGTCTTG-3′	9	3	6
OPA-16	5′-AGCCAGCGAA-3′	8	4	4
OPA-18	5′-AGGTGACCGT-3′	10	8	2
OPA-20	5′-GTTGCGATCC-3′	6	3	3
OPB-5	5′-TGCGCCCTTC-3′	5	3	2
OPB-8	5′-GTCCACACGG-3′	9	4	5
OPB-12	5′-CCTTGACGCA-3′	5	4	1
OPB-17	5′-AGGGAACGAG-3′	15	10	5
OPC-4	5′-CCGCATCTAC-3′	12	10	2
OPC-5	5′-GATGACCGCC-3′	9	6	3
OPC-9	5′-CTCACCGTCC-3′	8	4	4
OPC-11	5′-AAAGCTGCGG-3′	10	7	3
OPC-15	5′-GACGGATCAG-3′	7	6	1
OPC-18	5′-TGAGTGGGTG-3′	7	7	0
OPD-1	5′-ACCGCGAAGG-3′	8	5	3
OPD-13	5′-GGGGTGACGA-3′	11	8	3
OPD-15	5′-CATCCGTGCT-3′	10	4	6
OPD-16	5′-AGGGCGTAAG-3′	13	8	5
OPD-20	5′-ACCCGGTCAC-3′	9	3	6
Totals		178	113 (63%)	65 (37%)

amplified by 45 cycles of 94°C for 30 sec, 37°C for 1 min, 54°C for 30 sec, and 72°C for 2 min, followed by one cycle of 72°C for 15 min, and then held at 4°C. Products were analyzed by electrophoresis in a 1.2% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. Twenty primers were selected which produced several bands (typically five or more) which were judged to be bright, distinct, and easily scored for presence or absence of the bands (Table 1).

For each of the 20 primers, 25 RAPD-PCR amplification reactions were conducted simultaneously on a Perkin-Elmer thermocycler using the previous protocol. Four reactions were run for each bird: two reactions using the DNA sample from infected blood (or from blood taken on Day T for individual RD-6), and two reactions using the DNA sample from blood taken after treatment (Day T+9). The last reaction was a negative control which contained an aliquot of the reaction mix but no DNA. For each primer, all 24 reactions and the negative control were run bordered by a DNA ladder containing fragments in 100-base pair (bp) increments on each side.

RESULTS

We first examined bands which an investigator using RAPD-PCR for population genetics analyses would be likely to use (i.e., bright, sharp, easily scored). Next, we examined all products of the amplification process, regardless of brightness or clarity. All RAPD-PCR products from all primers were examined closely for any patterns which showed differences between replications of the same sample, or differences between Day T and Day T+9 samples of one individual.

Across the 20 primers, 178 bands were classified as being bright, distinct and easily scored; 113 (63%) of these were shared by all six individuals, and 65 (37%) showed some level of polymorphism among individuals (Table 1). We detected no case, either in the first or second stage of examination, in which an amplified product appeared in one of the replications from an infected sample and not in at least one of the replications of the post-treatment sample of the same individual. In the vast majority of cases, all the RAPD-PCR products (both high-quality scorable bands and the fainter or less distinct products) were found consistently across all four replications of infected and non-infected samples from an individual. Of the exceptions, all were repeatability failures between replications of the same sample, and none were consistent with the pattern expected from amplification of bands due to presence of parasite DNA. Only four of these exhibited the brightness and clarity of a marker acceptable for scoring in at least one replication (2.25% of the total scorable markers), falling within the range of 2–7% predicted by other RAPD researchers (Weeden et al. 1992). The negative control lanes occasionally revealed discernible amplification products. Six of these fragments corresponded to products identified as scorable bands in the Rock Dove samples (6/178 or 3.4%). These bands presumably would be unusable in a population genetics study without additional replications to verify that the markers in the population samples are not contamination artifacts.

DISCUSSION

The RAPD-PCR protocols and primers used in this experiment failed to amplify markers exclusive to

Haemoproteus even in samples which fall within previously reported ranges for high infection intensities in birds (e.g., 0.2–4.2 infected cells per 100 erythrocytes; Godfrey et al. 1987). The *Haemoproteus* genome by itself should contain enough priming sites for RAPD markers to be amplified by at least some of the primers. Ideally, these experiments would include a test of these primers on purified *Haemoproteus* DNA; however, this organism occurs almost exclusively within nucleated avian blood cells, making isolation and purification of parasite DNA extremely difficult if not impossible (T. J. C. Anderson, pers. comm.). If we assume that *Haemoproteus* has a haploid genome size equivalent to that of the confamilial parasite *Plasmodium* (Plasmodiidae; approximately 1.2×10^8 nucleotides; Sparrow et al. 1972), then using 20 primers we could expect to see at least six bands 500–3,500 nucleotides in length, based on the equations of Clark and Lanigan (1993). Clark and Lanigan (1993) also noted that this expected number is typically significantly lower than the mean number of bands actually amplified over a variety of primers.

Although the concentration of *Haemoproteus* DNA in each reaction was undoubtedly small, PCR is able to amplify target regions from single cells, and from target sequences which are present only once in a sample of 10^5–10^6 cells (Saiki et al. 1988). RAPD-PCR, which utilizes much shorter 10-nucleotide primers, appears to be more sensitive to variations in DNA concentration. Tests of the repeatability of RAPD banding patterns in successive dilutions of a DNA sample (Davin-Regli et al. 1995) found that priming of reactions occurred randomly at concentrations of DNA less than 1 pg μl^{-1} of reaction mixture. Reproducibility of banding patterns was found to be adequate in the range of 100 ng μl^{-1} to 10 pg μl^{-1}. For the highest intensity of *Haemoproteus* in this experiment (approximately 6.7 gametocytes per 100 erythrocytes), 1.6 pg μl^{-1} were present in the RAPD-PCR reaction, which may have precluded amplification of *Haemoproteus* fragments.

The mixing of samples does not seem to affect the efficiency of standard PCR; however, co-amplification requires that the two target sequences be present in approximately equimolar amounts (Chan et al. 1994). The study of mixed DNA templates in a RAPD-PCR reaction has revealed a great insensitivity for amplifying bands from template DNA which is in low concentration relative to a second DNA sample. When DNA samples from two species are mixed, RAPD bands from the sample at the lower concentration are never amplified when this sample is present in a proportion of 1:25 or less (Michelmore et al. 1991). Indeed, even when samples from two different individuals of the same species are mixed, some polymorphic bands will be amplified only when their respective genome is present in quantities several fold in excess of the competing genome (Williams et al. 1993). Further examination of the phenomenon of competition in RAPD-PCR amplification suggests that when samples from two divergent species are mixed, the relative complexity of the two genomes may determine which sample will produce amplification products. In Williams et al. (1993), DNA samples of both soybean (high complexity genome: 7.0×10^8 nucleotides) and cyanobacteria (low complexity genome: 4.0×10^6 nucleotides) were capable of generating RAPD markers when tested alone; when mixed, all detectable RAPD products came from soybean, even when cyanobacteria was present in 460-fold molar excess. This suggests the possibility of competition for priming sites, with the genome of higher complexity having more target sites with a greater degree of complementarity to the primer. Thus, assuming the avian genome is of relatively greater complexity than that of the protozoan parasite, we might expect to see only avian amplification products, even when the infection intensity is high. For our case, the *Haemoproteus* intensity was approximately 6.7 per 100 erythrocytes, and the relative concentration of *Haemoproteus* DNA to Rock Dove DNA was 1:498. This great bias is due to both the greater number of cells and to their greater genome complexity: the Rock Dove has a haploid DNA content of 2.0×10^9 nucleotides as compared to the 1.2×10^8 nucleotides of *Haemoproteus,* an almost 17-fold difference (Sparrow et al. 1972).

Our experiment tested the effect of the presence of *Haemoproteus* gametocytes on RAPD amplification results, however other hematozoan parasites also are found in avian blood samples. Various species of *Haemoproteus* were the most prevalent (19.5%) in studies of over 57,000 individual birds and 388 Nearctic species reviewed by Greiner et al. (1975), but species of *Leucocytozoon* were only slightly less prevalent (17.7%), followed by *Trypanosoma* (3.9%), *Plasmodium* (3.8%), microfilariae (3.1%), and *Haemogregarina/Lankesterella* (0.6%). Their prevalence can change drastically depending on the bird species and region studied (Greiner et al. 1975), as well as the season of the year (Bennett and Cameron 1974). Of greater concern will be the intensity of parasite in the blood sample. Although most reported mean intensities of infection in birds appear to be far below that which should merit concern (e.g., Fedynich et al. 1993, Fedynich and Rhodes 1995), erythrocyte infection rates of 50% have been reported as an extreme for Rock Doves (Gardiner et al. 1988). However, in these cases the proportion of parasite to host DNA in a sample would still be only 1:67. The amount of *Haemoproteus* DNA going into a 25-μl reaction would be approximately 12 pg μl^{-1}, which falls into the range necessary for repeatable amplification (Davin-Regli et al. 1995) and thus may merit exclusion from a RAPD analysis.

The results of this study indicate that the presence of *Haemoproteus* is unlikely to interfere with RAPD analysis of vertebrate DNA extracted from blood samples. However, two important points should be noted. First, whereas this study examined the effect of *Haemoproteus* gametocytes in the peripheral blood of the host, the schizogonic stage of *Haemoproteus* occurs in the endothelial tissue of the lungs, skeletal muscle, liver, spleen, and kidney, and is more frequently encountered than the erythrocytic developmental stage (Garnham 1966, Gardiner et al. 1988, Graczyk et al. 1994). Therefore, avian DNA samples extracted from these tissues may contain sufficient quantities of parasite DNA to be of concern to investigators using RAPD-PCR. And finally, although these results suggest that

it is difficult to amplify parasite DNA when mixed with vertebrate host DNA, parasitologists using RAPDs should note that the corollary is probably also true; namely, that very small amounts of vertebrate DNA may be all that is required to contaminate a sample of protozoan parasite DNA.

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