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Isolation of a Genetic Marker Linked to the *Bh* Gene by Genetically Directed Representational Difference Analysis of Closed Colony Japanese Quails

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ABSTRACT—Disappearance of the coloration pattern of the plumage and whole body hemorrhage is caused by an autosomal dominant *black at hatch* (*Bh*) mutation in the Japanese quail. In this study, we adopted genetically directed representational difference analysis (GDRDA) to obtain a genetic marker linked to *Bh* from our closed colony stock. A DNA pool of 10 wild-type (+/+) quails was subtracted from another DNA pool of 10 quails heterozygous for the *Bh* mutation (*Bh*/+). After analysis of 106 clones isolated by three series of GDRDA using three different restriction enzymes, one clone was shown to detect a restriction fragment length polymorphism (RFLP) between +/+ and *Bh*/+ quails. A genomic region flanking the polymorphic restriction site was cloned by inverse-PCR, and a PCR-RFLP maker linked to the *Bh* mutation was developed. The marker showed no recombination with the *Bh* mutation in 101 quails analyzed. This is the first report in which a genetic marker linked to a specific gene was successfully isolated by GDRDA using a closed colony population, and this strategy is expected to have wide applications in species whose genetic resources are not well established.

INTRODUCTION

Black at hatch (*Bh*) is an autosomal dominant mutation that changes the plumage coloration pattern in Japanese quails (Minezawa and Wakasugi, 1977). Heterozygous (*Bh/*+) and homozygous (*Bh/Bh*) embryos exhibit black and brown coating, respectively, whereas wild-type (+/+) embryos exhibit longitudinal black and yellow stripes in dorsal feather germs. *Bh/Bh* embryos die at an early stage of development (after 7 to 10 days of incubation) due to whole body hemorrhage (Kubota *et al*., 1995; Shiojiri *et al*., 1997). These findings indicated that the *Bh* gene plays important roles in the coloration pattern formation of the plumage and development of the vascular system in avian embryos. However, investigations into the *Bh* function in early embryogenesis has been inefficient due to the lack of appropriate genetic markers that can be used in early stage embryos. The only available method has been genotyping by the plumage phenotype after 10 days of incubation. Therefore, development of DNA-based genetic markers for the *Bh* gene is necessary to promote investigations of the *Bh* function in early embryogenesis, and is the first step for positional cloning of the gene itself. However, little genetic resources are available in Japanese quails, and it is impossible to perform linkage analysis in this species.

Representational difference analysis (RDA) is a genomic subtraction technique that can identify restriction fragment length polymorphisms (RFLPs) between two complex genomes (Lisitsyn *et al*., 1993). Reproducible and sensitive genomic subtraction was achieved by the use of representations of the genome, which is prepared by restriction digestion of the genome and PCR of the total digestion product using a universal adaptor and primer. Genetically directed RDA (GDRDA) is one of the modifications of RDA to isolate genetic markers linked to a specific gene, without any previous genetic resources. GDRDA has been successfully used to isolate genetic markers between two congenic strains (Lisitsyn *et al*., 1994) and to isolate markers linked to a specific locus in backcross and intercross animals of inbred strains (Lisitsyn

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et al., 1994; Toyota *et al.*, 1996; Higo *et al.*, 2000). In GDRDA, two pools of DNA are prepared so that one DNA pool will have the A/A or A/B genotype for a specific locus but random ones (C/C, C/D or D/D) for the rest and so that the other DNA pool will have the B/B genotype for the specific locus but random ones for the rest. Performing RDA using these two pools is known to result in isolation of RFLP markers linked to the specific gene, at least when the pools are prepared from crosses made from two inbred strains.

In this study, we applied GDRDA to the closed colony *Bh* stock, to isolate a genetic marker linked to the *Bh* mutation and to confirm that GDRDA works in a closed colony population. We successfully isolated one linked RFLP marker, and converted it into a PCR-RFLP marker by cloning the flanking sequence by the inverse-PCR method.

MATERIALS AND METHODS

Animals and DNA extraction

Bh mutant was originally found in a commercial stock of Japanese quail, Toyohashi stock (Suzukei Hatchery, Toyohashi, Japan), and the mutants were maintained at National Institute of Genetics, Mishima, Japan. A heterozygote, which had the background of the commercial stock, was mated with a wild-type quail of Iwata stock (Mizutani Hatchery, Iwata, Japan), and the F_1 heterozygotes were sister-brother mated. Our stock of *Bh* mutants has been maintained by random mating between heterozygotes for two decades. Genomic DNA was extracted from the thigh of 10 day-old embryos by the phenol/chloroform method after determination of genetic status for the *Bh* mutation by the coloration pattern of the plumage (Kubota *et al.*, 1995).

GDRDA

The following procedure of RDA was performed essentially as described (Lisitsyn *et al.*, 1993; Lisitsyn *et al.*, 1994; Toyota *et al.*, 1996). Genomic DNA was digested with a restriction enzyme (*Bam*HI, *Bgl*II or *Hin*dIII; Toyobo, Tokyo, Japan). After purification and quantification of the digestion product, DNAs of 10 *Bh*/+ and 10 +/+ quails were pooled as a tester and a driver, respectively. An R series adaptor (500 pmol) corresponding to the initial restriction enzyme (Lisitsyn *et al.*, 1993) was ligated to the tester and driver DNA (1.0 μg) with T4 DNA ligase (New England Biolabs, Beverly, MA) in 30 μl of reaction mixture. Three μl of each ligated product was amplified in a 400-μl PCR solution [1 μM R series primer (Lisitsyn *et al.*, 1993), 67mM Tris-HCl, pH 8.8, 4 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 100 μg/ml bovine serum albumin, 200 μM dNTP, and 15 U *Taq* polymerase (Toyobo)], by incubating at 72°C for 3 min and performing 25 cycles of PCR (each cycle consisting of denaturation at 95°C for 1 min and extension at 72°C for 3 min). After phenol extraction and ethanol precipitation, amplified DNA fragments (amplicons) were restricted with the initial enzyme, and purified by gel-filtration chromatography (CHROMA SPIN+TE-200; CLONTECH Laboratories, Palo Alto, CA).

A J series adaptor (Lisitsyn *et al.*, 1993) was ligated only to the tester amplicon. One μg of the tester amplicon and 40 μg of the driver amplicon were mixed up, precipitated with ethanol, and dissolved in 4 μl of 3 x EE (3 mM EDTA, 30 mM EPPS, pH 8.0). The mixture was denatured at 96°C for 10 min, and renatured at 67°C for 16 hr after adding 1 μl of 5 M NaCl. The renatured solution was diluted with 45 μl of 1 M NaCl. Renatured DNA in a 5-μl aliquot was amplified in a 400-μl PCR solution that contained a J series primer (Lisitsyn *et al.*, 1993) instead of an R series primer for 10 cycles. Single-stranded DNA was digested by adding 40 μl of 10 mM $ZnSO_4$ and 100 U of Mung Bean nuclease (NEB) to the PCR solution, and incubating at 30°C for 30 min. After phenol extraction and ethanol precipitation of the solution, one tenth of DNA was further amplified by PCR for 20 cycles.

The final product of the selective amplification (C1) was restricted with the initial enzyme, and purified by gel-filtration chromatography. An N series adaptor (Lisitsyn *et al.*, 1993) was ligated to the restricted and purified C1, and 200 ng of C1 with an N adaptor was mixed with 40 μg of the driver amplicon. The second cycle of competitive hybridization and selective amplification was performed to yield C2 as described above. After switching to the adaptor of C2 to the same adaptor as used for C1, the J adaptor, the third cycle was performed in a similar manner to yield C3.

Analysis of GDRDA products

The total C3 products using *Bam*HI, *Hin*dIII and *Bgl*II were cloned into the *Bam*HI, *Hin*dIII and *Bam*HI sites of pBluescript II (Stratagene, La Jolla, CA), respectively. After transformation of XL1Blue competent cells, 192 white colonies were picked up and cultured. Insert-positive clones were selected after PCR with T3 and T7 primers.

To examine whether obtained clones were independent and whether they really detected differences in the tester and driver amplicons, (a) the PCR solution of insert-positive clones, (b) the tester and the driver amplicons, and (c) individual amplicons prepared from 10 wild-types and 10 heterozygotes were dot-blotted onto Hybond-N+ nylon membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). On the other hand, the PCR solutions of insert-positive clones were restricted with an appropriate enzyme, run in 2.0% NuSieve GTG agarose (FMC, Rockland, ME) gel, and cut out from the gel. Preparation of fluorescein-labeled probes from the inserts in the gel, prehybridization, hybridization and signal detection were carried out using *Gene images* labeling and detection system (Amersham Pharmacia Biotech).

Southern blot analysis

Genomic DNA was restricted with an appropriate enzyme, and amplicons were prepared as above. Each one μg of amplicon was electrophoresed in 1.2% agarose gel, and each 8 μg of restricted genomic DNA was run in 1.0% agarose gel. The gel was blotted onto Hybond-N+ nylon membrane after denaturation. Preparation of the labeled probe, hybridization and signal detection were performed as described above.

Sequencing

PCR products using T3 and T7 primers were purified with ethanol precipitation. Each produced was sequenced with T3 and T7 primers using BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster, CA), and ABI PRISM 310 Gene Analyzer (PE Biosystems) according to manufacturer’s instruction.

Inverse PCR

Genomic DNA of a wild-type quail was digested with *Bgl*II, and self-ligation of the restriction product was performed with T4 DNA ligase (New England Biolabs). The ligation product was amplified with HDM1 (5'-AGGAACAAGGGCTGAACTGATTC-3') and HDM2 (5'-ACCTGATAAAGGAAGAAGAGTCGG-3') primers by 35 cycles of PCR (each cycle consisting of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 3 min) in 50 μl of reaction mixture with 200 nM each primer, 1 x GC buffer I (Takara Biochemicals, Kusatsu, Japan), 400 μM dNTP, and 2.5 U *LA Taq* polymerase (Takara). The PCR product was sequenced with HDM1 and HDM2 primers as described above, and two primers, NW1 (5'-CATCACAACCCAATATATTAAGAA-3') and NW2 (5'-GAACATTACAAGGCTTTGATTCA-3'), were designed.

PCR-RFLP analysis

With 5 ng of genomic DNA, PCR was carried out using NW1 and NW2 primers in 10 μl reaction [500 nM each primer, 1x *Taq* DNA polymerase buffer (Toyobo), 3 mM $MgCl_2$, 200 μM dNTP, and 0.25 U

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Taq polymerase (Toyobo)]. The thermal cycle consisted of 20 sec denaturing step at 94°C, 30 sec annealing step at 58°C, and 30 sec extension step at 72°C was repeated for 35 cycles. The PCR product was restricted with 5 U of *Hin*dIII, and run in 2.0% NuSieve GTG agarose gel.

RESULTS

Isolation of RFLP markers by GDRDA

Ten *Bh*/+ quails were randomly selected from *Bh*/+ quails, which were available in our closed colony stock and whose genotypes were known by their phenotype in the plumage. Ten +/+ quails were similarly selected. Using the *Bh*/+ DNA pool as the tester and the +/+ pool as the driver, three series of GDRDA were performed respectively with *Bam*HI, *Bgl*II and *Hin*dIII. The course of GDRDA using *Hin*dIII is shown in Fig. 1. The tester and driver amplicons presented the same smearing pattern when stained with ethidium bromide (Fig. 1, lanes 1 and 2), but differential (shown by an arrowhead) and other non-specific bands were gradually enriched along the three cycles of competitive hybridization and selective amplification (Fig. 1, lanes 3–5). A total of 192 clones in each series of RDA were analyzed for their independence and specific hybridization to the tester amplicon, and one clone (MHD1B3) obtained from the *Hin*dIII series of GDRDA was found to detect specific hybridization to the tester amplicon (Table 1). MHD1B3 was further used to detect RFLPs in Southern blots of the electrophoresed amplicons of 10 *Bh*/+ and 10 +/+ quails. MHD1B3 detected two bands (600 bp and 700 bp) in all the 10 *Bh*/+ amplicons and one band (700 bp) in all the 10 +/+ amplicons (Fig. 2). From the *Bam*HI and *Bgl*II series of GDRDA, 36 and 54 independent clones were obtained, respectively, but no clone showed specific hybridization.

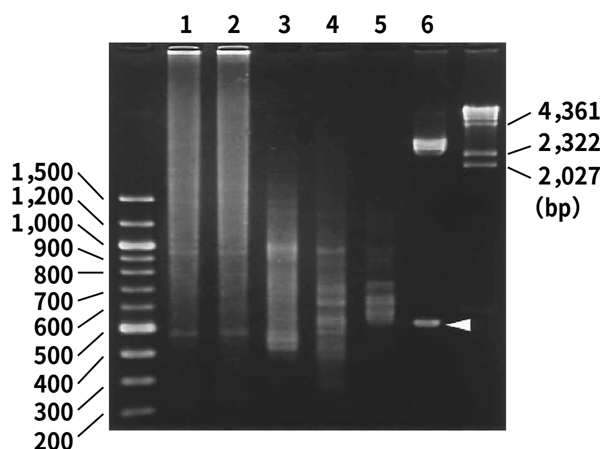

Fig. 1. Course of GDRDA using *Hin*dIII restriction enzyme. Samples were run in 0.9 % agarose gel and stained by ethidium bromide. Lanes 1 and 2: 500 ng of driver (wild type) and tester (heterozygote) amplicons; lanes 3, 4 and 5: 200 ng of the product after the first (C1), second (C2) and third (C3) cycles; lane 6: 100 ng of MHD1B3 clone. The arrowhead indicates the band derived from MHD1B3 insert. The 3 kb band in lane 6 was derived from pBluescript II.

Design of PCR-RFLP primers and genotyping

To use the RFLP detected by MHD1B3 with small quantity of DNA, the *Hin*dIII polymorphism was converted into a PCR-RFLP marker by inverse-PCR strategy (Triglia *et al.*, 1988). First, the sequence of MHD1B3 was determined (GenBank Accession No. AB042522). Two primers, HDM1 and HDM2, facing toward outside of clone MHD1B3 were then designed based on the sequence. Genomic DNA of *Bh*/+ quails was digested with eight different restriction enzymes, respectively, and Southern blot analysis was performed using MHD1B3 as a probe. Since MHD1B3 detected a band of approximately 3k bp in DNA digested with *Bgl*II (data not shown), inverse-PCR was performed using self-ligated DNA after *Bgl*II digestion with HDM1 and HDM2 primers (Fig. 3A). Both ends of an amplified 3k bp product were sequenced (data not shown), and it was noted that the wild-type allele had a 5'-AAGTTT-3' sequence, instead of 5'- AAGCTT-3', a *Hin*dIII recognition site, at one end of MHD1B3.

A pair of new primers, NW1 and NW2, were designed so that they amplify a 220 bp fragment spanning the polymorphic *Hin*dIII site. Digestion of the PCR product of the *Bh* mutant allele was expected to yield 120 and 100 bp fragments, while that of the wild-type allele was to remain uncut. To confirm that this strategy works, DNA of +/+, *Bh*/+ and *Bh*/*Bh* quails were amplified (Fig. 3B, lanes 1–3), and cut with *Hin*dIII (Fig. 3B, lanes 4-6). The +/+ quail displayed the 220 bp band only, the *Bh*/+ displayed the 220 bp band and the combination of 120 and 100 bp bands, and the *Bh*/*Bh* quail displayed the

Table 1. Summary of the clones isolated by the three series of GDRDA.

	No. of clones		
	analyzed	independent	differential*
*Bam*HI	192	36	0
*Bgl*II	192	54	0
*Hin*dIII	192	26	1

* Clones that gave positive hybridization signals in the amplicons of the tester pool and 10 individual *Bh*/+ quails, and negative signals in those of the driver pool and 10 individual +/+ quails.

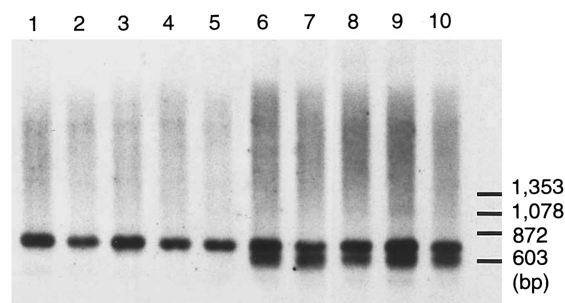

Fig. 2. Tight linkage of MHD1B3 to *Bh* genotypes. One μg of amplicon was electrophoresed in a 1.2% agarose gel and blotted onto nylon membrane. Lanes 1–5: amplicons prepared from individual +/+ quails; lanes 6-10: amplicons prepared from *Bh*/+ quails. No recombination was observed in a total of 101 quails.

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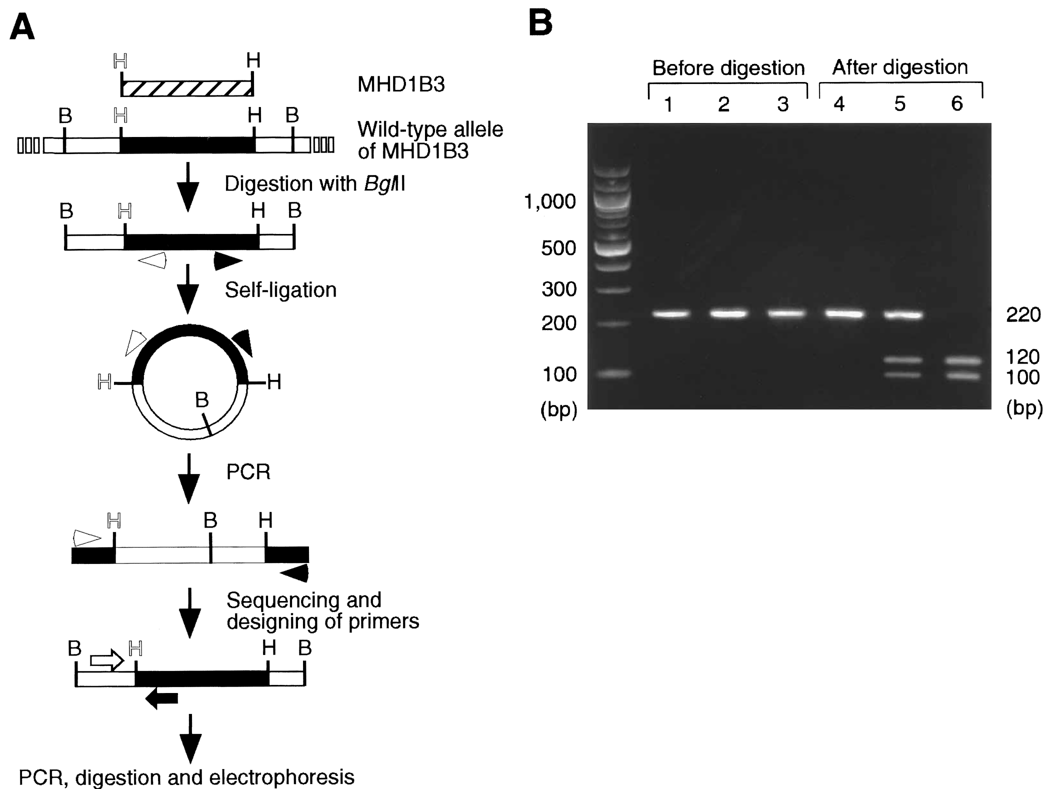

Fig. 3. Conversion of MHD1B3 marker into a PCR-RFLP marker. (A) Strategy of inverse-PCR and designing of PCR primers. B and H are the *Bgl*II and the *Hin*dIII sites, respectively. Based on the sequence of MHD1B3, a pair of primers, HDM1 (open arrowhead) and HDM2 (closed arrowhead) were designed. A wild-type genomic DNA was digested with *Bgl*II, by which an allelic fragment overlapping MHD1B3 was yielded (It had been confirmed by Southern blot of the *Bgl*II digest of a wild-type genomic DNA probed with MHD1B3: data not shown), and self-ligated. Then the fragment was amplified with the primers by PCR and sequenced with the same primers. Based on the determined sequence, a pair of new primers, NW1 (open thick arrow) and NW2 (closed thick arrow), were designed so that they amplify a fragment spanning the polymorphic *Hin*dIII site (open H). Using these primers, genomic DNA was amplified by PCR and then digested with *Hin*dIII. The digest was electrophoresed and genotype of the samples was judged by its band pattern. (B) Result of *Bh* genotyping. Lanes 1–3: PCR products of each genotype; lanes 4–6: the *Hin*dIII digests of the PCR products. Lanes 1 and 4 are derived from +/+. Lanes 2 and 5 are derived from *Bh*/+. Lanes 3 and 6 are derived from *Bh*/*Bh*. Samples were run in 2.0% NuSieve GTG gel and stained by ethidium bromide.

combination only. Further, this PCR-RFLP method was applied to 101 quails (57 +/+, 41 *Bh/+* and 3 *Bh*/*Bh* quails), whose genotypes were previously known, and no recombination was observed in this population.

DISCUSSION

In this study, we obtained an RFLP marker linked to the *Bh* mutation by performing GDRDA in a closed colony stock of *Bh* quails. This is the first report in which GDRDA was successfully applied to isolate genetic markers using such animals. Since many mutant animals are maintained in closed colonies, often without appropriate genetic resources, this strategy seems to have a wide range of applications.

Our stock of *Bh* mutants was established by mating a heterozygote of the original closed colony stock, Toyohashi, with another closed colony stock, Iwata, and sister-brother mating of the F_1 heterozygote. Therefore, *Bh* mutant allele in our stock is derived from the Toyohashi stock, and wild-type allele of the *Bh* gene in our stock is derived from the Iwata stock, which is considered to be phylogenetically distant from the original *Bh* stock. This could be one of the important reasons why we succeeded in the application of GDRDA directly to a closed colony stock, not to offsprings prepared for linkage analysis. If this is the major reason, it is necessary to make F_1 animals with a genetically distant closed colony stock or inbred strain and to obtain F_2 animals by sister-brother matings of heterozygotes to succeed in GDRDA. However, even in this case, we do not have to stick to inbred strains, as is shown in this study.

The marker isolated showed no recombination in the 101 *Bh* quails whose genotype had already been determined by the plumage phenotypes. As mentioned above, our stock of *Bh* mutants has been maintained by random mating in a closed colony for two decades, which corresponds to approximately 60 generations. Chromosomal segment that shows linkage disequilibrium is thus expected to be very small, and the marker has a good chance to be very close to the *Bh* gene. The small size of chromosomal segment with linkage disequilibrium with the *Bh* gene also explains the small yield of clones linked to the *Bh* locus, only one clone in three series of GDRDA.

The RFLP marker, which can be used in Southern blot,

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obtained by GDRDA was converted into a PCR-RFLP marker after cloning flanking sequence by inverse-PCR. This simple and reliable marker will accelerate the investigations into the function of the *Bh* gene on abnormal pattern formation of the plumage pigmentation and blood vessels in *Bh* homozygotes at early stages of development. This marker will also enable positional cloning of the *Bh* gene once large-insert library of the quail is established.

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