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Production of Interspecific Germline Chimera between Quail and Chicken Utilizing the Cells from Central Disk of Blastoderm and Germinal Crescent Region

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Production of interspecific germline chimera between quail and chicken was carried out using the cells from the central disk (CD) of the area pellucida in quail blastoderm (stage X) and the germinal crescent region (GCR) of quail embryo (stage 7–8). The cells of CD and GCR were dispersed and injected into the subgerminal cavity of chicken blastoderm (stage X). Injected eggs were incubated for 7 days or to hatching. To detect quail genomic DNA, the polymerase chain reaction was used. In day–7 embryos, quail DNA was detected in 4 gonads and 6 brains from 55 survived embryos received quail CD cells, and 9 gonads and 8 brains from 68 survived embryos received quail GCR cells. Quail DNA was also detected from eggs or semen of adult female or male chicken hatched from eggs received quail CD or GCR cells. CD and GCR cells as the donors showed the possibility to produce the interspecific germline chimera but the necessary improvement and the immunoreactive problem were suggested.

INTRODUCTION

For the preservation of endangered species in bird like as the crested ibis, *Nipponia nippon*, production of interspecific germline chimeras (Naito *et al.*, 1991; Ono *et al.*, 1996; Ono *et al.*, 1998a, b; Fujihara, 1999; Li *et al.*, 2002) will be one of the useful methods. In order to facilitate the improvement of this method, gene markers, which can detect the donor cells in recipient, are very important tools. There are some gene markers for interspecific chimeras between quail and chicken (Ono, 2001; Li *et al.*, 2001). Also, we have used another primer set for chicken ovalbumin to identify the donor chicken DNA in the recipient quail by polymerase chain reaction (PCR) in the previous study (Soh *et al.*, 2002). In the present study, we have designed the primer set for quail ovalbumin and tried to produce interspecific germline chicken chimeras with quail cells as the donor.

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MATERIALS AND METHODS

Donor cells

Fertilized quail eggs, which were freshly oviposited and incubated for 26–29 h, were swabbed with 70% ethanol. CD of the area pellucida in blastoderm and GCR were cut off and the cells were dispersed in phosphate buffered saline (PBS) by pipetting. These cells were resuspended in Dulbecco's MEM to become 500 cells/μl after centrifugation (400 x g, 10 min) and removing supernatant.

Recipient eggs and injection

Fresh and fertile chicken eggs swabbed with 70% ethanol were stored for 2–3 days at room temperature with marked equatorial plane up. A window 5–7 mm in diameter was opened at the mark. Then, 1 μl of donor cell suspension (500 cells) was injected into the subgerminal cavity of recipient embryo using drawn micropipette (50–70 μm outer diameter). Each window was closed with an adhesive tape. Injected eggs were incubated for 7 days or to hatching at 37.7 °C and 70% relative humidity.

Preparation of genomic DNA and progeny test

Gonads and a part of brain were collected from the embryos at day–7. Semen was collected from an adult male chicken hatched from eggs received CD cells. The blastoderms were collected from fertilized eggs freshly oviposited by female chickens, which hatched from eggs received CD or GCR cells, artificially inseminated with chicken semen. Genomic DNA samples were extracted by the conventional method using phenol–chloroform. DNA concentration was determined by spectrophotometer at 260 nm.

The progeny test was performed with quail hens by artificial insemination of the semen collected from chimeric male chicken.

PCR

To detect quail genomic DNA by PCR, a primer set for ovalbumin cDNA (GenBank #X53964) was designed: primer Q1, 5'–CAGAGGCTGGAGTGGATGCTA–3' (sence) and primer Q2, 5'–TATTACTCTGTGTAAGGGAAGGGTGAAGT–3' (antisence). After an initial denaturation step (95 °C, 10 min), the PCR was performed in reaction mixture (10 μl) containing 0.2 μM each of the primers, 1 x PCR buffer, 0.2 mM dNTPs, 0.25 U AmpliTaq Gold polymerase (Perkin Elmer Applied Biosystems, Foster, CA, USA), and 10 ng of DNA template. The amplification step consisted of 40 cycles using denaturation (95 °C, 30 sec), annealing (60 °C, 30 sec), and extension reaction (72 °C, 2 min) before a final extension step of 10 min at 72 °C. The PCR products, of which predicted size was 309 bp (Fig. 1), were analyzed by electrophoresis on 1.5% agarose gels. The PCR products amplified with quail genomic DNA was checked by a restriction enzyme Bfa I (New England BioLabs, Beverly, MA, USA), and the resulting products were analyzed by electrophoresis on 1.5% agarose gels. To compare the intensities of the signals, 10, 1 and 0.1 ng of quail genomic DNA with 10 ng of chicken DNA were used for template. Chicken genomic DNA was used as the negative control.

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1081 ·········c agaggctgga gtggatgcta ctgaagaatt tagggctgac catccattcc
1141 tcttctgtgt caagcacatc gaaaccaacg ccattctcct ctttggcaga tgtgtttctc
1201 cttaaaaaga agaaaatata aagagtctgt ccctttcagc aagacccaga gcactgtagt
1261 atcaggggta aaatgaaaag catgttctct gctgcatcca gactttataa agttagggcc
1321 ttatctagga aaaaaaaaaa aaaaaagaag atcatatgag aacaggtgca cttcaccctt
1381 cccttacaca gagtaata·· ·········· ·········· ·········· ··········
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Fig. 1. Nucleotide sequence of the amplified region of quail ovalbumin cDNA (GenBank #X53964). Underlined sequences are the primer regions.

RESULTS

Verification of the PCR product

The PCR product of the expected size (309 bp) was amplified from quail genomic DNA, and when the template decreased, the intensity of the signal weakened (Fig. 2). Chicken genomic DNA was not amplified with the primer set for quail. The 309 bp quail PCR fragment was cleaved with Bfa I, which yielded the expected fragments of 236 and 73 bp.

Detection of quail DNA in day–7 chicken embryos

A total of 123 chicken eggs received quail CD cells. The survival rate of embryos at day–7 was 45% (Table 1). Quail DNA was detected from 4 embryos in the gonad and 6 embryos in the brain as revealed by PCR analysis. A total of 122 chicken eggs received quail GCR cells. The survival rate of embryos at day–7 was 56% (Table 1). Quail DNA was detected from 9 embryos in the gonad and 8 embryos in the brain. The intensities of the signals of these embryos received CD or GCR cells were estimated same as or less than 1/100 comparing with positive control (10 ng of quail DNA).

Table 1. Detection of quail DNA from chimeric chicken embryos at day–7 by PCR

Donor	Cells	Recipient[1]	Injected eggs	Survived embryos at day–7	Quail DNA detected in gonad	Quail DNA detected in brain	Quail DNA detected in both gonad and brain
Quail	Central disk of blastoderm[1]	Chicken	123	55 (45%)	4 (7%)	6 (11%)	0 (0%)
Quail	Germinal crescent region[2]	Chicken	122	68 (56%)	9 (13%)	8 (12%)	4 (6%)

[1]Fertilized eggs freshly oviposited were used.
[2]Fertilized eggs incubated for 26–29 h were used.

Detection of quail DNA in semen or eggs of adult chickens and progeny test

Four chickens hatched from 15 eggs those were injected with quail CD cells. Three chickens grown up and they revealed two females and one male after reaching to sexual maturity. Quail DNA was detected from a part of the semen of the male (Fig. 3) or an egg of one female artificially inseminated with chicken semen (Table 2, Fig. 4). Three chickens hatched from 22 eggs those were injected with quail GCR cells. Two chickens grown up and they revealed females after reaching to sexual maturity. Quail DNA was also detected from three eggs of one female artificially inseminated with chicken semen

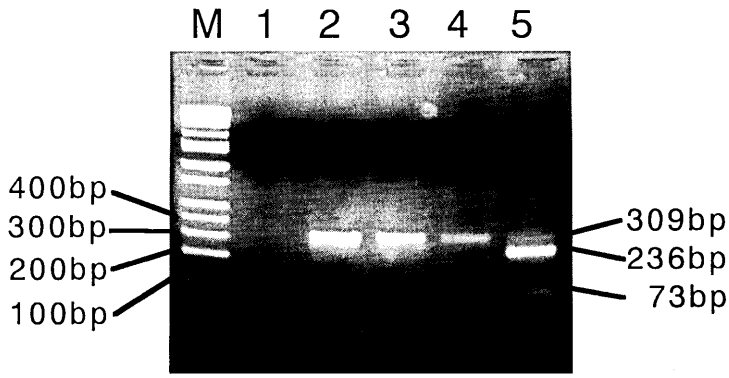

Fig. 2. Amplified PCR products from quail genomic DNA. M: Molecular size marker. 1: 10 ng of chicken genomic DNA (negative control). 2: Quail and chicken genomic DNA (10 ng:10 ng). 3: Quail and chicken genomic DNA (1 ng:10 ng). 4: Quail and chicken genomic DNA (0.1 ng:10 ng). 4: Digestion of the amplified product with Bfa I. Digested fragments appeared 236 bp and 73 bp.

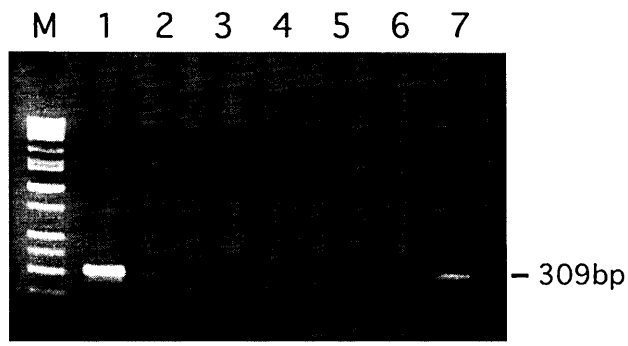

Fig. 3. Detection of quail DNA from chicken semen. M: Molecular size marker. 1: 10 ng of quail genomic DNA (positive control). 2: 10 ng of chicken genomic DNA (negative control). 3–7: Sperm samples (10 μl) taken from one ejaculated semen. Quail DNA was detected from only one sample (lane 7).

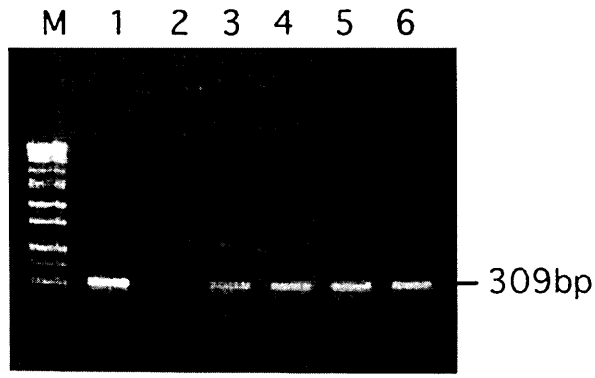

Fig. 4. Demonstration of detected quail DNA from four chicken eggs. M: Molecular size marker. 1: 10 ng of quail genomic DNA (positive control). 2: 10 ng of chicken genomic DNA (negative control). 3–6: Blastoderms of chicken eggs from which quail DNA was detected.

Table 2. Detection of quail DNA from putative chimera chicken hens

Donor cells	Individual No.	No. of eggs	Detected quail DNA
Quail CD[1]	62	44	1 (2.3%)
Quail CD[1]	64	38	0 (0%)
Quail GCR[2]	70	42	3 (7.1%)
Quail GCR[2]	73	33	0 (0%)

[1]Central disk of blastoderm.
[2]Germinal crescent region.

(Table 2, Fig. 4). The semen of the chimeric male chicken was artificially inseminated to quail hens. A total of 2030 eggs were collected and incubated, but no quail and 54 hybrids hatched. In addition, the chimeric male chicken had bent left leg and the two chimeric female chickens died suddenly within two months after sexual maturity.

DISCUSSION

The Quail ovalbumin has the sequence of amino acid shorter than that of chicken as described in the previous study (Soh *et al.*, 2002). This difference has been considering in designing the primer set. The primer set for quail ovalbumin was able to amplify PCR products with quail genomic DNA but not chicken genomic DNA in the present study. Other available primer sets to detect interspecific chimera between quail and chicken have also been reported (Ono, 2001; Li *et al.*, 2001). As well, the peafowl-specific primers designed from cyt b gene sequences have been used to detect the interspecific chimera between peafowl and chicken (Xi *et al.*, 2002).

The chicken CD cells in blastoderm at stage X (Eyal–Giladi and Kochav, 1976) and the cells of GCR at stage 7–8 (Hamburger and Hamilton, 1951) include pre–PGCs (Ginsburg and Eyal–Giladi, 1986, 1987, 1989; Kagami *et al.*, 1997; Tagami and Kagami, 1998; Kagami *et al.*, 2000; Naito *et al.*, 2001) and PGCs (Eyal–Giladi *et al.*, 1981; Urven *et al.*, 1988; Han *et al.*, 1994), respectively. As the donor cells, they showed the possibility to produce the interspecific germline chimera in the previous study (Soh *et al.*, 2002). As well, the quail CD cells and GCR cells also showed the possibility to be useful donor cells for germline chimeras since the donor quail DNA was detected in the gonad of the recipient chicken embryo and in the semen or eggs of the adult recipient chicken as revealed by PCR in this study.

The survival rate at day–7 and hatchability of manipulated chicken embryos seems to be better than those of quail embryos (Soh *et al.*, 2002). But obtained germline chimeras were not so lot and their chimera rates were also very low as same as previous study (Soh *et al.*, 2002). Any quail chicks have not hatched, however, the semen of the chimeric male chicken has been inseminated to quail hens. Further studies are needed to make necessary improvement as suggested in previous study (Soh *et al.*, 2002).

There were other problems of interspecific chimeras, e.g., the sudden death and the abnormal body of chimeric chickens. All chimeric hens had been dead within two months after sexual maturity and the chimeric male chicken has bent left leg. These problems may be caused from the immunoreaction to donor cells of different species. The donor cells may have to be settled in the germline restrictedly or the somatic chimera rate has to be reduced as possible.

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