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ORIGINAL PAPER

Construction of cytoplasmic molecular markers distinguishing *Danio rerio* from *Gobiocypris rarus* at high identity domains based on MP-PCR strategy and Sybr Green I detection

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Abstract To distinguish the cytoplasm of *Danio rerio* from that of *Gobiocypris rarus*, we cloned *G. rarus COXI* and constructed cytoplasmic molecular markers at the high identity domains of *COXI* by mutated primer PCR (MP-PCR for short). Then Sybr Green I was used to detect the single amplicon. As a result, we succeeded in getting the cytoplasmic molecular markers, G.M COXI and Z.M COXI, by MP-PCR strategy. They were used to detect the sperm-derived mtDNA in the sexual hybrid embryos (*D. rerio* ♀ × *G. rarus* ♂) before the sphere stage. In the present study, all results demonstrate that MP-PCR approach and Sybr Green I detection are feasible to construct the molecular markers to identify genes that shared high identity.

Keywords COXI · *Danio rerio* · *Gobiocypris rarus* · MP-PCR · Sybr Green I detection

Introduction

Nuclear transfer between two fish species, i.e., interspecies cloning, offers us a useful tool to study nucleo-cytoplasmic interaction and nuclear reprogramming (Zhu and Sun 2000; Sun et al. 2005). *Danio rerio* has been an excellent model for developmental genetic studies for its short sex-maturity cycle, high reproductive capacity, and transparent eggs etc (Grunwald and Eisen 2002; Key and Devine 2003). *Gobiocypris rarus*, a special local species in China, not only shares aforementioned advantages, but also has many unique traits for laboratory study, such as typical eurytherm and high adaptation (Wang 1995), toxicity testing (Qun-Fang et al. 2002) and sensitivity to hemorrhagic virus of grass carp (Wang et al. 1994a, 1994b) etc. Such advantages underlie *G. rarus* to become another good model for developmental and genetic studies (Wang and Cao 1997). Since the cloned embryos, derived from the nuclei of *D. rerio* and the enucleated eggs of *G. rarus*, have the same nuclei but different cytoplasm compared with normal *D. rerio* embryos, it becomes an ideal model to study nucleo-cytoplasmic interaction and nuclear reprogramming. In recent study, researchers focus the limited success of cloned embryos on an original viewpoint—the mitochondrial function (Hiendleder et al. 2005). To well understand the component of mitochondrion in such cloned embryos, we cried for an mtDNA marker to distinguish *D. rerio* from *G. rarus*.

Since PCR technique was invented by Mullis (Mullis et al. 1986), many kinds of PCRs had been developed, such as inverse PCR (Ochman et al. 1988), touch-down PCR (Don et al. 1991) and long distance PCR (Barnes 1994) etc. They innovated mainly in reaction program and PCR reaction system, but rarely in primers except for mutation require. When we need to distinguish two genes that shared high identity, to construct good molecular markers became a hard nut to crack. However, amazing effect will give birth, when we alter

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a few bases in the primers correctly. Because appropriate change in primers can eliminate primer hairpin structure, reduce the Tm distance between two primers and enhance the PCR specificity. Such changed primer PCR was called mutation PCR (MP-PCR for short) by us. It can relieve the agony to fumble the optimal reaction condition in PCR process. In the present work, we not only constructed molecular markers to distinguish the cytoplasm of *D. rerio* and *G. rarus* by MP-PCR strategy, but also applied Sybr Green I detection to detect the single amplicon.

Materials and methods

Experimental materials

D. rerio and *G. rarus* were provided by our pisciculture station in Institute of Hydrobiology. The sexual hybrid embryos (*D. rerio* ♀ × *G. rarus* ♂) were produced by in vitro fertilization. Embryos were incubated in Holtfreter's solution at 28°C, and were collected at seven developmental stages (zygote, 256-cell, sphere, 50% epiboly, bud, 15-somite, early larva) as described by Kimmel et al (1995).

Isolation of total DNA

D. rerio and *G. rarus* total DNA (TDNA for short) and the sexual hybrid embryos (*D. rerio* ♀ × *G. rarus* ♂) TDNA were extracted according to standard phenol-chloroform method (Sambrook et al. 2001).

Cloning of *G. rarus* COXI

The *COXI* sequences of *Carassius auratus* (AB006953), *Carassius carassius* (AY714387), and *Sarcocheilichthys variegates* (AB054124) were downloaded from NCBI website (National Center for Biotechnology Information). After aligning them with Clustal X software (Thompson et al. 1997), we designed two homologous primers—$COXI_{for}$ (CTACCCTACCTGTGGCAATC) and $COXI_{rev}$ (GAGTGGTTATGTGGCTGGCT) by Primer Premier 5 software. PCR was performed by using 0.5 U of Taq DNA polymerase (BioAsia, Shanghai, China), 10 pmol primers and 50 ng of template DNA in a volume of 25 μl. The reaction process was 94°C 5 min; then 94°C 60 s, 55°C 60 s, 72°C 120 s, 30 cycles; 72°C 5 min, 4°C hold on GeneAmp PCR System-9700 (Applied Biosystems).

Application of MP-PCR strategy for constructing cytoplasmic molecular markers

Since *D. rerio* and *G. rarus* share 82.4% identity on *COXI* genes, it is difficult to find appropriate primers to distinguish them. MP-PCR strategy was introduced according to the following three principles. First, the mutated bases must be in the identical regions; Second, the mutated bases cannot locate at the 3-terminal of primers; Third, to reduce the T_m distance between two primers, G or C is altered to A or T in the primer with higher GC content and A or T is altered to G or C in the primer with lower GC content. Thus, G.COXI (ATAGTCATCTCTACTGAAGAT) and D.COXI (GCGGTCAATTCAACTGACAGA) were chosen at their high identity domain. The bases needed to mutate are indicated with italic letters and the identical bases between two primers are underlined. When we changed A_7 and T_{10} all to G in G.COXI but C_6 to A and A_7 to T in D.COXI, *G.M Primer* (ATAGTC*G*TC*G*C-TACTGAAGAT) and *D.M Primer* (GCGGT*A*T-ATTCAACTGACAGA) were given birth. The annotation of marks was the same as described above. The share forward primer was P1 (TTATTGTATGAGCCCACCAC). P1 and *G.M Primer*, special to *G. rarus* COXI, were called G.M COXI for short, while P1 and *D.M Primer*, special to *D. rerio* cytoplasmic *COXI*, were called Z.M COXI for short. PCR was performed by using 0.5 U of Taq DNA polymerase (BioAsia, Shanghai, China), 10 pmol of G.M COXI or Z.M COXI primers, and 50 ng of template DNA in a volume of 25 μl. The reaction process was 94°C 5 min; then 94°C 30 s, 53°C 30 s, 72°C 60 s, 30 cycles; 72°C 5 min, 4°C hold. PCR products were separated by electrophoresis on 1.0% (w/v) agarose gel.

Sybr Green I direct detection

2× SYBR Green I dye buffer (Applied Biosystems) was added into each 5 μl finished PCR reaction mixture and mixed well. Then 2 μl aliquot was dropped onto glass slice and observed under a 420 nm wavelength fluorescence microscope.

Tracing the sperm mitochondrial DNA fate of sexual hybrid embry with cytoplasmic molecular markers

To prove the validity of cytoplasmic molecular markers constructed by MP-PCR technique, the sexual hybrid embryos (*D. rerio* ♀ × *G. rarus* ♂) were chosen to trace the destiny of sperm mitochondrial DNA. PCR was

performed with G.M COXI or Z.M COXI primers in a total volume of 25 μl, respectively. The PCR mixture and the PCR program were the same as mentioned above. Meanwhile, *GAPDH* was chosen as an endogenous reference for each PCR reaction, whose primer sequences were GTGTAGGCGTGGACTGTGGT and TGGGAGTCAACCAGGACAAATA. The PCR products were also detected by Sybr Green I.

Results

Cloning and characterization of *G. rarus* COXI

A fragment (about 1.6 kb) was amplified from *G. rarus* TDNA with $COXI_{for}$ and $COXI_{rev}$ (Fig. 1). Then, the fragment was sub-cloned into pMD18-T vector and sequenced. The sequencing result indicated that it was just *G. rarus COXI* (the Accession No. was AY879113 at Genbank). Its sequence was 1.55 kb in length, coding 516 amino acids. The alignment comparison of *COXI* with *D. rerio* (AC024175) shows that they share 82.4% identity (Fig. 2), indicating that it is intractable to design sound primers to distinguish them.

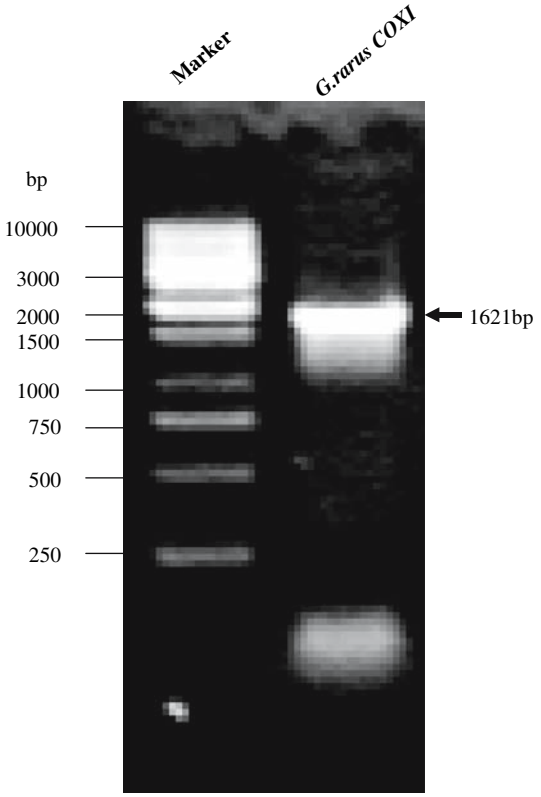

Fig. 1 Cloning of *G. rarus COXI* Marker-GeneRuler™ 1 kb DNA Ladder (Fermentas co., America). *G. rarus COXI*—the *COXI* of *G. rarus* was amplified with $COXI_{for}$ and $COXI_{rev}$ primers. The amplicon is 1621 bp as indicated with arrow

Feasibility of cytoplasmic molecular markers based on MP-PCR technique and Sybr Green I dye detection

Primers analysis with Primer Premier 5 software shows that G.COXI and D.COXI have serious dimmers and hairpin structures. Whereas, the analysis of G.M Primer and Z.M Primer by MP-PCR strategy indicates that they are sound primers according to common primer design principle (Innis et al. 1990). Moreover, the mutated bases located at the consensus region help to enhance the specificity of PCR. As expected, G.M COXI and Z.M COXI could well distinguish *G. rarus* from *D. rerio*, with only the partial fragment of *COXI* (613 bp) (Fig.3A). Therefore, we could directly detect the cytoplasm derived from *G. rarus* or from *D. rerio* just by Sybr Green I dyeing with G.M COXI or Z.M COXI primer, respectively. The result of Sybr Green I detection was in agreement with that of electrophoresis (Fig. 3B), indicating that Sybr Green I detection was an easy and efficient method to detect the single amplicon.

The destiny of the sperm-derived mtDNA in the sexual hybrid embryo

All the total DNA concentrations of sexual hybrid embryos (*D. rerio* ♀ × *G. rarus* ♂), at different development stages, were normalized by *GAPDH*. The PCR result shows that sperm-derived mtDNA could be detected before the sphere stage with Z.M COXI. After the sphere stage, sperm-derived mtDNA faded away beyond detection. However, ovum-derived mtDNA could be detected at all development stages with G.M COXI and their amplicons were the same intensity. On the contrary, the intensity of amplicons with G.M COXI was obviously feebler than that with Z.M COXI before the sphere stage. The reason was the quantitative difference of sperm-derived mtDNA and ovum-derived mtDNA. The result of the detection by Sybr Green I was consistent with that by agarose electrophoresis (Fig. 4).

Disscussion

Cytoplasmic molecular markers distinguishing *D. rerio* from *G. rarus* based on MP-PCR strategy were successfully achieved, indicating that MP-PCR strategy is a sound method to construct molecular marker, especially for high identity genes. In fact, we could not distinguish *D. rerio* from *G. rarus* with the primers without mutation (data not shown). Besides for

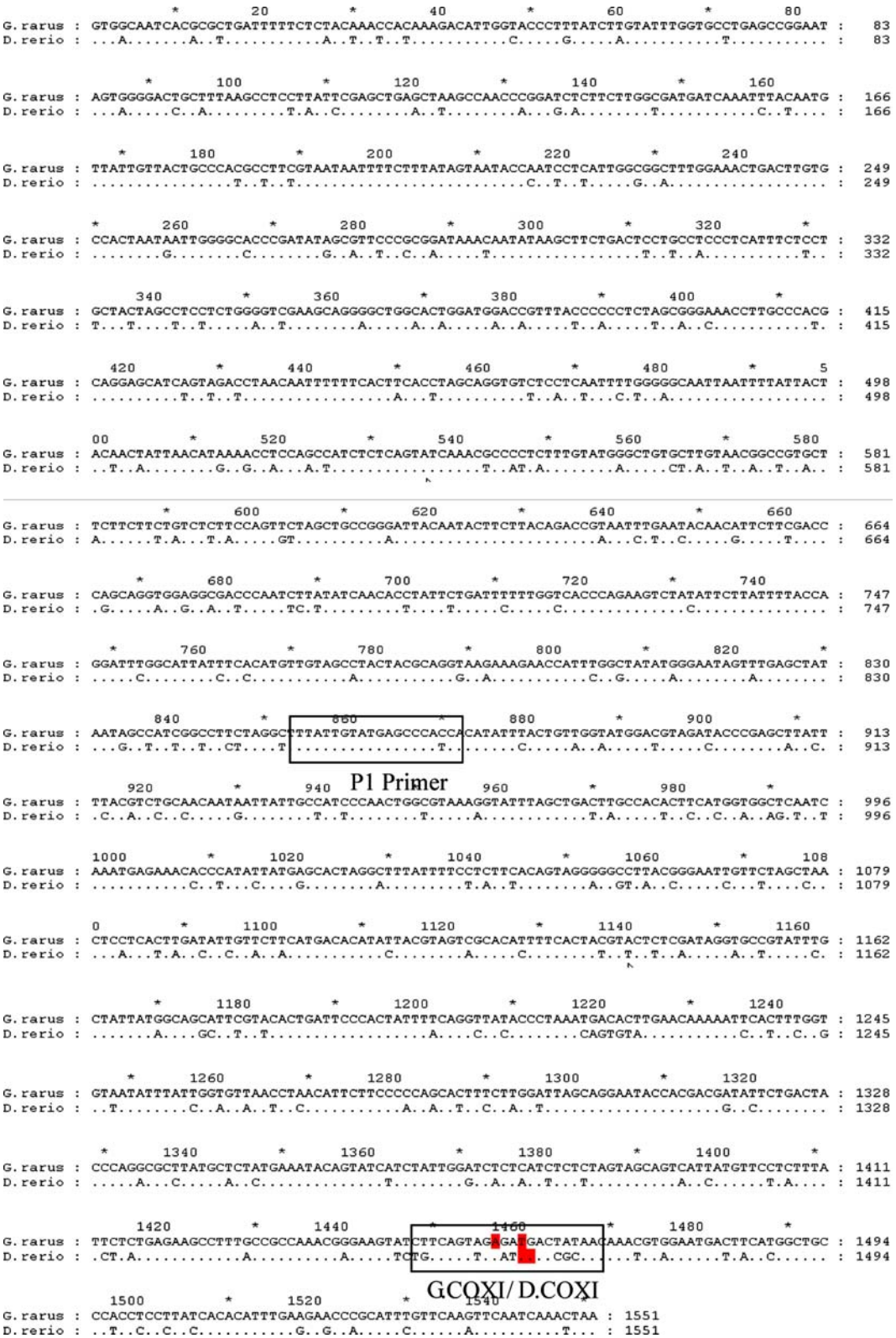

Fig. 2 Alignment comparison of *G. rarus COXI* with *D. rerio COXI* The length of *G. rarus COXI* is 1551 bp with 82.4% identity compared to *D. rerio COXI*. The consensus bases are indicated with dots and the bases mutated by MP-PCR strategy are marked with shade. P1 primer, D.COXI and G.COXI are showed with panes

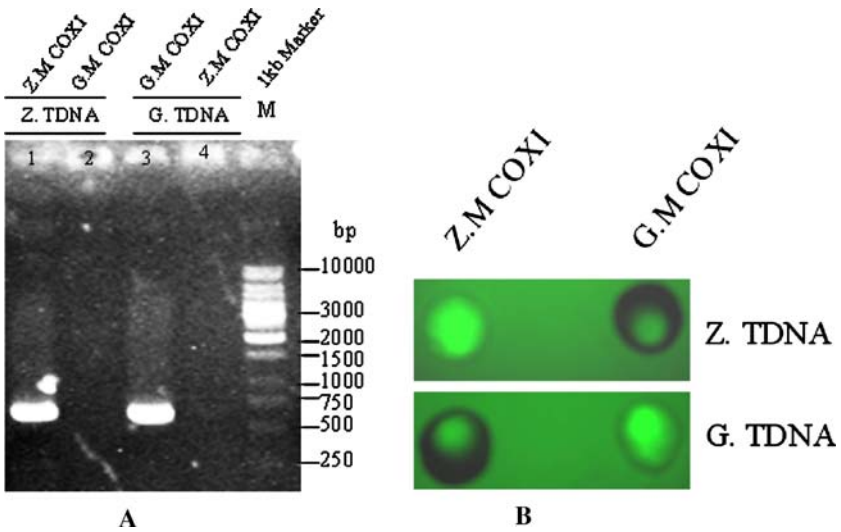

Fig. 3 Agarose electrophoresis analysis and Sybr Green I direct detection of amplicons with G.M COXI and Z.M COXI in *G. rarus* and *D. rerio* total DNA. (**A**) PCR was performed with G.M COXI and Z.M COXI in *G. rarus* total DNA (G. TDNA) and *D. rerio* total DNA (Z TDNA). Lanes 1, 2 were PCR results in Z TDNA with Z.M COXI and G .M COXI primers, respectively; Lanes 3, 4 were PCR results in G. TDNA with G.M COXI and Z.M COXI primers, respectively; M-GeneRuler^{TM} 1 kb DNA Ladder (Fermentas co., America). (**B**) PCR products were detected with Sybr Green I. The Primers (Z.M COXI and G.M COXI) and the templates (Z. TDNA and G. TDNA) were indicated above

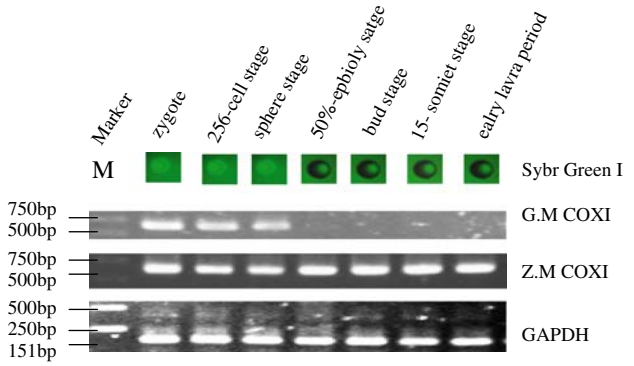

Fig. 4 The detection to the sperm mtDNA and ovum mtDNA in the sexual hybrid embryo (*D. rerio* ♀ × *G. rarus* ♂) Lanes from left to right were different development stages of the sexual hybrid embryos; Lanes from up to down were Sybr Green I detection and agarose electrophoresis detection of the sexual hybrid embryo TDNA with G.M COXI, Z.M COXI and GAPDH primers, respectively

molecular markers, MP-PCR can also be applied for producing correct protein on the condition that the mutated bases were samesense mutation.

There are two kinds of cytoplasms, *G. rarus* derived from its sperm and *D. rerio* derived from its ovum, in the sexual hybrid embryo (*D. rerio* ♀ × *G. rarus*♂). Thus, Z.M COXI and G.M COXI can be used to track the destiny of each cytoplasm. In present study, the result shows that the sperm-derived mtDNA existed before the sphere stage; Henceforth, it faded away beyond detection. This finding is in agreement with our previous study on the sexual hybrid embryo (*Carassius auratus* ♀ × *Cyprinus carpio* ♂) (Sun et al. 2005). Additionally, G.M COXI and Z.M COXI can be used to evaluate the quality of sexual hybrid embryo (*D. rerio* ♀ × *G. rarus* ♂) based on mtDNA heteroplasmy. In the sexual hybrid embryos before the sphere stage, when the amplicons (613 bps) were both produced by G.M COXI and Z.M COXI, the pseudo-development of embryo could be excluded.

The phenomenon of mtDNA heteroplasmy in the sexual hybrid embryo (*D. rerio* ♀ × *G. rarus* ♂) was also reported in mammal somatic cell cloned embryos (Steinborn 2002; Han et al. 2003; Inoue et al. 2004). When exploring the development of cloned embryos derived from transgenic common carp (*Cyprinus carpio*) nuclei and goldfish (*Carassius auratus*) enucleated eggs, we also found that the mtDNA of donor and receptor co-existed in the cloned embryos before blood-circulation (Sun et al. 2005). Not come singly but in pairs, the same phenomenon was found in inter-species cloned embryos, such as chicken–rabbit cloned embryos (Liu et al. 2004). In our ongoing studies, G.M COXI and Z.M COXI is used not only to analyze the mtDNA heteroplasmy in the cloned embryos derived from the nuclei of *G. rarus* and the enucleated eggs of *D. rerio*, but also to track the destiny of donor-derived mtDNA. All that will help to unveil the mechanism of nucleo-cytoplasmic interaction and nuclear reprogramming in such cloned embryos.

Since the only one amplicon (613 bp) was produced by G.M COXI and Z.M COXI, the direct detection method with Sybr Green I could judge the cytoplasm's derivation without electrophoresis. In previous report, ethidium bromide was used to direct stain the PCR products of the SCAR markers for purity testing of F1 hybrid seed in chili pepper (Capsicum annuum) (Jang et al. 2004). However, ethidium bromide had the suspicion that it caused cancer. On the contrary, Sybr Green I has many advantages, such as strong signal, low setting and low concentration usage (Tuma et al. 1999). Thus, direct Sybr Green I detection without electrophoresis was much safer and more sensitive than ethidium bromide.

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