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Developmental Expression Pattern

Characterization of novel genes expressed specifically in the sexual organs of the planarian *Dugesia ryukyuensis*

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ABSTRACT The planarian *Dugesia ryukyuensis* reproduces both asexually (fissiparous) and sexually (oviparous) and can switch from the asexual mode to the sexual mode. By feeding with mature *Bdellocephala brunnea* oviparous worms, the fissiparous worms, which do not possess sexual organs, can be converted to fully sexualized worms in a process termed sexualization. As sexualization proceeds, the sexual organs are formed uniformly and five stages (stages 1–5) of the process have been identified histologically. In order to clarify the sexualization process, we attempted to isolate the genes expressed specifically at stage 5 by the differential display method. We isolated five genes expressed in the testis and two genes expressed in the yolk gland, which is an organ specific to sexualized worms. By BLAST search, one of the testis-specific genes was coded as testis-specific *α-tubulin* and two yolk gland-specific genes are similar to ribose-phosphate pyrophosphokinase I and F-box/SPRY-domain protein 1. *Drs1, Drs2 and Drs3* were expressed in spermatocytes and spermatids from the early stage of spermatogenesis and *Drs4 and Drs5* were expressed in spermatogonia, spermatocytes and spermatids. These genes are useful markers for elucidating the sexualization process.

KEY WORDS: *planarian, sexualization, point-of-no-return, testis, yolk gland*

Living organisms have established unique reproductive systems to multiply and sustain their species. Two types of reproduction evolved, namely, asexual and sexual (Bell, 1982). Planarians (Platyhelminthes, Turbellaria, Tricladida) reproduce both asexually (fissiparous) and sexually (oviparous) and they can switch from one mode of reproduction to the other (Curtis, 1902; Curtis and Schulze, 1924; Kenk, 1937; Kenk, 1940). An experimental system was established in which sexually mature worms of the oviparous species *Bdellocephala brunnea* are fed to fissiparous *Dugesia ryukyuensis* worms in order to elucidate the mechanisms underlying the conversion from an asexual mode of reproduction to a sexual mode of reproduction (Kobayashi *et al*., 1999; Hoshi *et al*., 2003). This experimental sexualization is apparently triggered by a sexualizing substance(s) contained in oviparous planarians (Grasso and Benazzi, 1973; Benazzi, 1973; Grasso *et al.,* 1975; Sakurai, 1981; Kobayashi *et al.*, 1999). The sexualization process is divided histologically into five stages (stages 1–5). As sexualization proceeds, the fissiparous worms form mature sexual organs. A pair of ovaries appears behind the head at stage 1 and mature as sexualization proceeds. The primordial testes, which are located on the dorsal side, emerge at stage 3, spermatocytes and spermatids can be observed at stage 4 and many sperm are detectable in the testes at stage 5 (Kobayashi and Hoshi, 2002). Differentiation of the copulatory apparatus starts at the end of stage 3 and a genital pore opens on the ventral surface at stage 4. The yolk glands appear at stage 4 and develop with sexualization. It was demonstrated that worms in stages 1 and 2 of the sexualization process could return to the fissiparous state if feeding on *B. brunnea* was stopped. However, worms from stage 3 onward could not return to the fissiparous state. Therefore, a time-point, referred to as the point-of-no-return, exists between stages 2 and 3 (Kobayashi *et al.,* 1999). This suggests that from the point-of-no-return onward, worms produce sexualizing substance(s) in their own bodies in order to maintain their sexualized condition (Kobayashi *et al*., 2002). Accordingly, it is important to understand the acquisition of sexuality and the sexualizing events in detail after the point-of-no-return.

Abbreviations used in this paper: Drs, Dugesia ryukyuensis sexual organ specific gene.

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During the sexualizing process, the development of the ovary and the opening of the genital pore can be observed, but development of the testes and other sexual organs could be detected only histologically. It is difficult to categorize worms into each stage by external observation and especially after the point-of-no-return the only external feature is the genital pore at stage 4. *Dryg* was the first cDNA characterized as being expressed in the yolk gland, which is one of the specific organs for oviparous worms, of worms at stage 5. *Dryg* expression started in neoblast-like cells in worms at stage 3, so this gene is a good marker for analyzing the sexualization process after the point-of-no-return (Hase *et al.,* 2003). The genes expressed in worms at stage 5 are molecular markers of the sexualizing process.

Recently, to characterize the sexualization process by use of the molecular markers, the worms at stage 5 expressed sequence tag database (the sexualized planarian EST-DB: http://planaria.bio.keio.ac.jp/planaria/) was constructed and opened as an online resource (Ishizuka *et al*., in press). It contains 3,077 expressed clusters, but this is not enough to cover all the genes expressed in the worms at stage 5. For a detailed study, many of the markers for sexual organs are essential. In this study, to obtain the trace of specific genes in the worms at stage 5 using the differential display method, we tried to isolate genes specific for worms at stage 5. We successfully isolated seven genes that are expressed in the testes and yolk glands, both of which differentiate after the point-of-no-return.

Results

Isolation of specific genes at stage 5

Seven specific genes (*Drs1–7*) in worms at stage 5 were obtained by the comparison between fissiparous worms and worms at stage 5 with the differential display method. These genes were examined for stage 5-specificity by Northern blot hybridization using RNAs extracted from fissiparous worms and worms at stage 5 (Fig.1A). The results revealed a single band detected specifically in worms at stage 5. The sizes of of *Drs1*, *Drs2*, *Drs3*, *Drs4, Drs5, Drs6* and *Drs7* mRNAs were predicted to be 0.7 kb, 1.1 kb, 2.0 kb, 2.1 kb, 0.9 kb, 1.4 kb and 1.4 kb, respectively. Their full-length cDNAs were isolated and sequenced. The cDNA sequences have been deposited in GenBank under accession numbers AB284772 (*Drs1*), AB284773 (*Drs2*), AB284774 (*Drs3*), AB284775 (*Drs4*), AB284776 (*Drs5*), AB284777 (*Drs6*) and AB284778 (*Drs7*). *Drs2*, *Drs3*, *Drs4, Drs5, Drs6 and Drs7* encoded a predicted polypeptide of 272, 534, 584, 239, 316 and 352, amino acid residues, respectively. Since *Drs1* did not appear to encode a significant open reading frame (ORF), this gene may not be translated into a polypeptide and may belong to a non-coding RNA gene (ncRNA), which could act as the developmental timer of protein expression.

Expression stage of Drs1-7 *in sexualization*

RT-PCR was performed to determine stage-specificity during sexualization (Fig. 1B). Amplifications of *Drs1*, *Drs2*, *Drs4 and Drs6* were recognized only in the RNA extracted from worms at stage 5. On the other hand, the *Drs5* and *Drs7 and Drs3* fragments were amplified slightly at stage 3 and stage 4, respectively and increased as sexualization proceeded. These genes were expressed after the point-of-no-return and they should be good gene markers for sexualization.

Histological analysis of Drs1-7 *on sexualized worms*

To analyze the spatial expression patterns of *Drs1–7,* whole-mount *in situ* hybridization was performed with worms at stage 5. The expression of *Drs1–5* was detected specifically as two lines on the dorsal side, which could be regions of the testes (Fig. 2A–E). The expression of *Drs6* and *Drs7* was detected specifically in the region of the yolk glands, which is along the ventral nerve system (Fig. 3A and B).

To investigate this expression more precisely, section *in situ* hybridization was performed. *Drs1, Drs2 and Drs3* were expressed in the testes at the level of spermatocytes and spermatids (Figs. 2F, G and H) and were revealed by hematoxylin and eosin

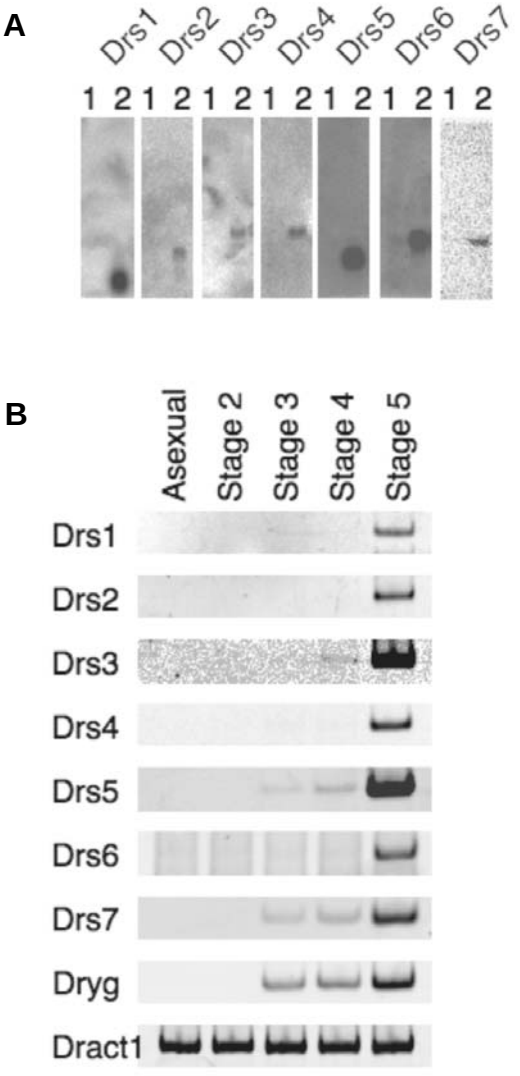

Fig. 1. Sexualized worm-specific expression of *Drs1–7*. (A) *Northern hybridization with the fissiparous worm (1) and the worm of stage 5 (2).* **(B)** *RT-PCR for stage-specificity during the sexualization process (fissiparous, stage2, stage3, stage4 and stage5).*

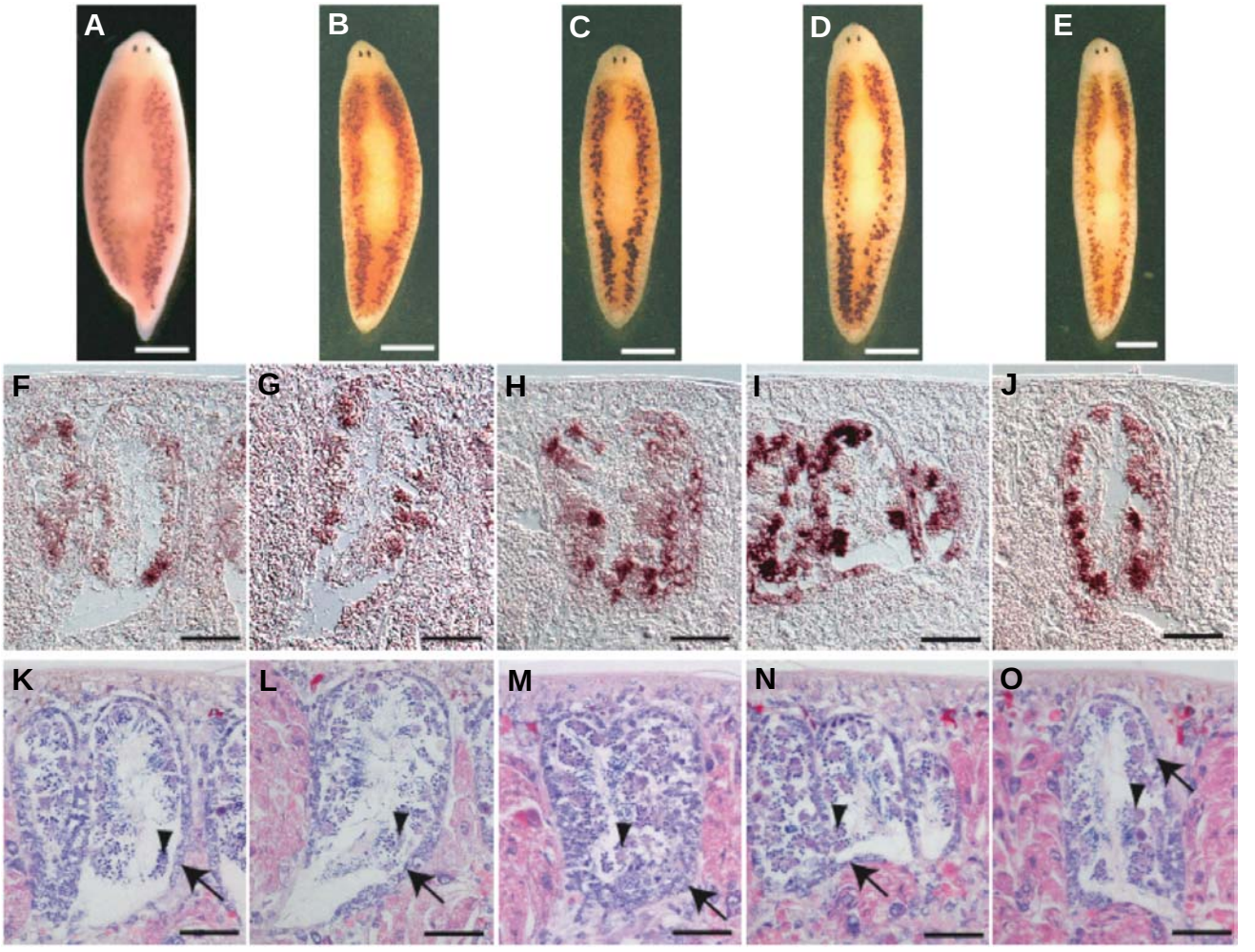

Fig. 2. Histological analysis of testis-specific genes *Drs1–5*. (A–E) *Whole mount* in situ *hybridization with* Drs1-5 *as probe.* **(F–J)** *Section* in situ *hybridization. Planarian sagittal sections hybridized with* Drs1-5. **(K–O)** *The neighboring sections stained with HE. Spermatogonia are shown by the arrow and spermatocytes are shown by the arrowhead. Scale bars, (A-E) 1 mm; (F-O) 50 μm.*

(HE) staining (Figs. 2K, L and M). No signal was detected in the spermatogonia. The signals of *Drs4* and *Drs5* were expressed in the testes at the levels of spermatogonia, spermatocytes, spermatids and developing spermatids (Fig. 2I and J), as revealed by HE staining (Fig. 2N and O). The signals of *Drs6* and *Drs7* appeared in whole yolk gland cells (Fig. 3C and D) and were revealed by HE staining (Fig. 3E and F).

Homology search of Drs1-7

By the BLAST search, four of them (*Drs1, Drs2, Drs4 and Drs5*) were not included in any database and were recognized as novel genes. Genes of *Drs3, Drs6 and Drs7* were annotated. By searching for them in the sexualized planarian EST-DB, it was found that *Drs3* was highly similar to DrC-00131, annotated testes-specific α-tubulin isoform (*E*-value < 6*E*-151). *Drs6* was highly similar to DrC-00052, annotated human ribose-phosphate pyrophosphokinase I (*E*-value < 4*E*-85) and *Drs7* was highly similar to DrC00667, annotated mouse F-box/SPRY-domain protein 1 (*E*-value < 5*E*-31). *Drs6* and *Drs7* were matched completely to DrC-00052 and DrC00667, respectively and are known genes. While this EST-DB included 7 clusters of α-tubulin gene, using the Clustal W program to analyze the differences between *Drs3* and DrC-00131, it was matched with only 53.5% identity at the amino acid level and *Drs3* is a novel gene in the *D. ryukyuensis* genome. DrC-00052 and DrC00667 were present in 12 genes and 9 genes in the EST-DB, suggesting that they are expressed abundantly, but the others were not included in the EST-DB. Therefore, these results showed that the differential display method is a practical method for cloning the many markers for oviparous worms (or at stage 5).

Discussion

In this study, we could identify seven genes, including five novel genes."*Drs3* is annotated as α-tubulin and the extremely homologous region includes: the diagnostic portion, which is conserved exclusively within the α-tubulin subclass; the potential GTP-binding site (GGGTGSG) required for the polymerization of the α- and β-chains (Sternlicht *et al.,* 1987). As the sexualized planarian EST-DB showed seven clusters of encoded α-tubulin,

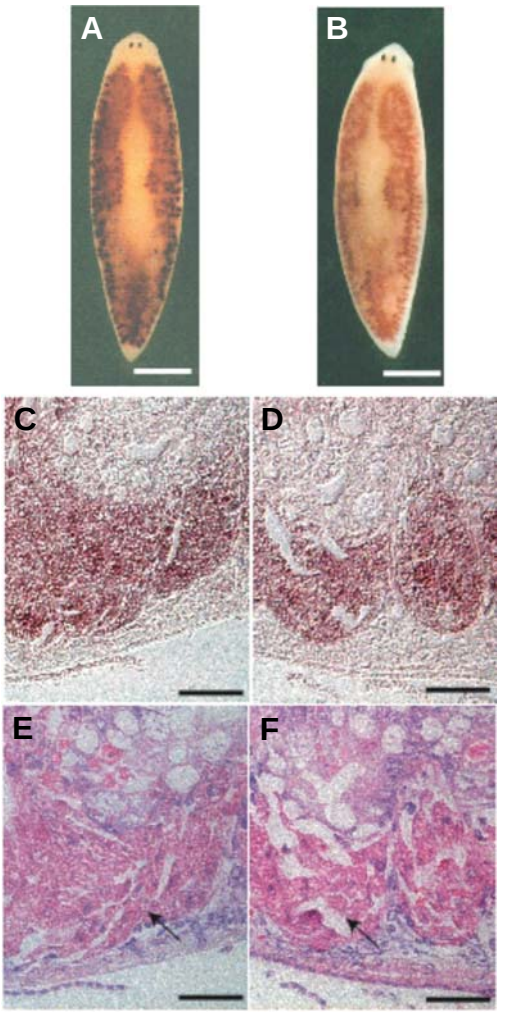

Fig. 3. Histological analysis of yolk gland-specific genes *Drs6* and *Drs7*. (A,B) *Whole mount* in situ *hybridization with* Drs, Drs7 *as probes, respectively.* **(C,D)** *Section* in situ *hybridization. Planarian sagittal sections hybridized with* Drs6 *and* Drs7*, respectively.* **(E,F)** *The neighboring sections stained with HE. Yolk gland cells are shown by the arrow. Scale bars: (A,B) 1 mm, (C-F) 50 μm.*

the tubulin multi-gene family exists in the *D. ryukyuensis* genome, but *Drs3* is different from the seven cluster genes. Few data are available about the α-tubulin genes in the Tricladida. Molecular cloning involves an α-tubulin gene in *Schmidtea polychroa* as *Sp*Tub-1 (AF516884) and a partial α-tubulin cDNA in *Schmidtea mediterranea* (AF112360). *Sp*Tub-1 is a testis-specific tubulin isoform and is expressed in spermatogonia, spermatocytes and spermatids (Salvetti *et al.,* 2002, Simoncelli, *et al*., 2003), while the α-tubulin in *S. mediterranea* is expressed in the epithelium but not in testes (Sanchez and Newmark, 1999). The expression pattern of *Sp*Tub-1 is very similar to that of *Drs3*, but using the Clustal W program to analyze the differences between *Drs3* and other α-tubulins, the sequence similarity with the α-tubulin in *S. mediterranea* is only 45.71%. And the C-terminal region of *Drs3* contained a long insertion sequence. These findings showed that *Drs3* is not a homolog for the *Sp*Tub-1 gene. Almost all animals express some testis-specific tubulin, which is a major protein in the sperm tail for sperm motility. The *Drs3* product could also act in sperm tail movement.

One of the annotated genes was a ribose-phosphate pyrophosphokinase (RPPP; EC 2.7.6.1) isoform, which is catalyzed from MgATP and ribose-5-phosphate in the synthesis of virtually all nucleotides, as well as being an important regulator of rates of the *de novo* pathways of purine and pyrimidine nucleotide synthesis. In mammals, one of two RPPP isoforms is detected in almost all tissues and the other isoform is detected in the testes. In the planarian, the yolk gland has a function to supply the egg. Then, the yolk gland-specific RPPP isoform might be expressed.

Drs7 is annotated as F-box/SPRY-domain protein 1, which is known as a synaptic protein in several animals. Many F-box proteins are target recognition subunits of SCF (Skp, Cullin, F-box) ubiquitin-ligase complexes. And FSN-1, the encoded F-box/SPRY-domain protein 1 in *Caenorhabditis elegans,* is required in presynaptic neurons for the restriction and/or maturation of synapses and provides a localized signal to attenuate presynaptic differentiation. It is not understood how the nervous system regulates the development or function of the yolk gland in the planarian.

Though *Drs1, Drs2* and *Dr 4* stage 5-specific expression genes, *Drs1* and *Drs2* were transcribed only in spermatocyte and *Drs4* was transcribed in spermatogonia and spermatocyte. Since spermatogonia appeared from stage 3 in this sexualization system, *Drs4* might not be related to spermatogenesis, but might be related to the maintenance of testes.

Earlier, we constructed the sexualized planarian EST-DB containing 3077 expressed clusters of worms at stage 5 (Ishizuka *et al*., in press). In this study, we obtained the seven specific genes in worms at stage 5, including five unknown genes using the differential display method. Almost none of the 3077 clusters in EST-DB can be used as a marker for sexual organs and the rate of cloning markers for sexual organs with the differential display method is higher than that for the EST-DB. It was shown that this method is more effective for isolating genes that are transcribed at a low level, which may nevertheless be important.

Experimental Procedures

Animals

We used fissiparous worms and worms from stages 2–5 of the OH strain of *D. ryukyuensis*. The sexualizing system followed Kobayashi's method (Kobayashi *et al*., 1999). The animals were maintained at 20°C in dechlorinated tap water.

Isolation of stage 5-specific cDNA

Total RNA was isolated from fissiparous worms or worms at stage 5 that were starved for 1 week. To identify the genes that are expressed specifically in worms at stage 5, we employed differential display techniques using RNA Image Kits (GenHunter). A partial cDNA of *Drs1-7* was obtained using the primers H-T11G and H-AP5, H-T11C and H-AP4, H-T11A and H-AP4, H-T11C and H-AP9, H-T11A and H-AP9, H-T11C and H-AP14 and H-T11G and H-AP12, respectively. The full lengths of these genes were obtained by screening a cDNA library in Lambda ZAP II (Stratagene) or using a SMART RACE kit (Clontech) following the manufacturer's instructions.

Northern hybridization

For Northern blot analysis (Sambrook *et al.,* 1989), we separated 7.5 g of total RNAs on 1% agarose gel containing formaldehyde and trans-

ferred them to a positively charged nylon membrane (Pall Biodyne B). Northern blot hybridization was carried out under the following conditions: hybridization, 4 x SSC, 0.12 mg/ml salmon sperm DNA, 0.2% SDS, 5 x Denhardt's solution, 50% formamide at 42°C for 16 h; washing, 2 x SSC-0.1% SDS at 50°C for 10 min, 0.2 x SSC-0.1% SDS at 50°C for 1†h, repeated once. The cDNAs were labeled with [α-^{32}P]dCTP (3000 Ci/mmol) using a random prime labeling system (Amersham Pharmacia Biotech) for hybridization probes. After washing, the blots were analyzed using a BAS5000 Bio-Image Analyzer (Fuji Photo Film). *Dract1* (GenBank accession number AB292462, actin in *D. ryukyuensis*) was used as internal control and *Dryg* (GenBank accession number AB090278), which is expressed in the yolk gland, was used as positive control.

In situ hybridization and histology

Whole-mount *in situ* hybridization was performed in accordance with the protocols described by Umesono *et al*. (1997) and Agata *et al.* (1998). Section *in situ* hybridization was performed as described by Kobayashi *et al.* (1998). Sense and antisense riboprobes were prepared by *in vitro* transcription using RNA polymerase with digoxigenin-UTP (Roche). For histology, the sections were stained with HE.

Polymerase chain reaction and cDNA cloning

RT-PCR analysis was performed using total RNAs from fissiparous worms and worms from stages 2–5 and specific primers as follows:

Drs1	F	5'-CCATCAGGGACGAATATTCCAG-3'
	R	5'-TGAGTATCTTTAGTTGAAATAC-3',
Drs2	F	5'-TCATATGGAATCAAATATTACACCAGG-3',
	R	5'-CACGTTGAGATTATGCGATACTGCATAG-3',
Drs3	F	5'-CTCGTCTGTTGAGTTAACAGATC-3',
	R	5'-TGTGAAGTGGCCTCGTGCAAAG-3',
Drs4	F	5'-AGAAAAACTTTGAAGATGACACCTC-3'
	R	5'-AAGTATCTCCTGTTCCTTCATTGCCACG-3'
Drs5	F	5'-GACAGTAACTGTTGGAACGTC-3'
	R	5'-CGTATTTCTGGCATATCTAAGAGC-3'
Drs6	F	5'-GTCATCCGCTCGTATCACAGCAG-3'
	R	5'-GGCCACACGTTCTGCACTGGTAAC-3'
Drs7	F	5'-GACATCATTGCCGATATAAT-3'
	R	5'-ATGACTCAAATACGAGCATA-3'
Dryg	F	5'-TTGGACGGATTTCTATTCTATGCC-3'
	R	5'-CACTGGGTTTCTGATCTCTTTCG-3'
Dract1	F	5'-CAGAAATGAGAACAGCATCAGC-3'
	R	5'-CAATTCCAGGTCCAGATTGTC-3'

The annealing temperature was 55°C for *Drt1*, *Drt5*, *Drt6* and *Drt7* and 65°C for *Drt2*, *Drt3* and *Drt4*. In all, 36 cycles were performed. Control reactions were performed in the absence of reverse transcripts.

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Mammalian Reproduction and Development

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